

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12N 15/19, C07K 14/52, C12N 5/10, A61K 38/19</b>		A2	(11) International Publication Number: <b>WO 96/25498</b> (43) International Publication Date: <b>22 August 1996 (22.08.96)</b>
(21) International Application Number: <b>PCT/US96/02492</b> (22) International Filing Date: <b>14 February 1996 (14.02.96)</b>		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(30) Priority Data: 08/388,779 15 February 1995 (15.02.95) US 08/591,070 9 February 1996 (09.02.96) US		Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(71) Applicant (for all designated States except US): AMGEN INC. [US/US]; Amgen Center, 1840 Dehavenilland Drive, Thousand Oaks, CA 91320-1789 (US).			
(72) Inventor; and			
(73) Inventor/Applicant (for US only): ELLIOTT, Steven, G. [US/US]; 1040 Golden Crest, Newbury Park, CA 91320 (US).			
(74) Agents: ODRE, Steven, M. et al; Amgen Inc., Amgen Center, 1840 Dehavenilland Drive, Thousand Oaks, CA 91320-1789 (US).			
(54) Title: <b>MPL LIGAND ANALOGS</b>			
(57) Abstract			
<p>Mpl ligand analogs having one or more changed glycosylation sites as compared to a naturally occurring mpl ligand sequence of a corresponding number of amino acids are disclosed. The invention also relates to DNA sequences encoding said mpl ligand analog, recombinant plasmids and host cells for analog expression, and therapeutic compositions including such analogs.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	L1	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LX	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Sweden
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

- 1 -

## MPL LIGAND ANALOGS

### Field of the Invention

5        The present invention relates to mpl ligand analogs having at least one changed O- or N-linked glycosylation site. The invention also relates to DNA sequences encoding these mpl ligand analogs, and recombinant plasmids and host cells for analog expression.

10

### Background of the Invention

15       MGDF, or megakaryocyte growth and development factor, is a recently cloned cytokine that appears to be the major regulator of circulating platelet levels. See Bartley, T.D. et al., *Cell* 77:1117-1124 (1994); Lok, S. et al., *Nature* 369:565-568 (1994); de Sauvage, F.J. et al., *Nature* 369:533-538 (1994); Miyazake, H. et al., *Exp. Hematol.* 22:838 (1994); and Kuter, D.J. et al., *PNAS USA*, 91:11104-11108 (1994). MGDF as described in Bartley, T.D. et al., *Cell* 77:1117-1124 (1994), is also referred to as thrombopoietin (TPO), mpl ligand, and megapoietin. Herein, the term "mpl ligand" will be used generically to refer to all polypeptides that activate the mpl receptor, including TPO and MGDF. The mpl receptor is a cell surface protein that, upon activation, leads to production and/or development of megakaryocytes and platelets. See WO 92/07074.

20       "Mpl ligand analogs" are polypeptides that differ from native sequences in a way that affects the number, location or type of carbohydrate linkage sites. Such polypeptides are one aspect of the present invention. Mature native human mpl ligand is a protein

25

30

35

A-337B

- 2 -

having 332 amino acids in total. The sequence of this protein (attached to a 21-amino acid long leader sequence) and the corresponding cDNA are shown in FIG. 1 herein (SEQ. ID NOS.: 1 and 2).

5 Recombinant mpl ligand produced in both Chinese Hamster Ovary (CHO) and *E. coli* cells has been demonstrated to have a biological activity of specifically stimulating or increasing megakaryocytes and/or platelets *in vivo* in mice, rats and monkeys. See 10 e.g., Hunt, P. et al., *Blood* 84(10):390A (1994). Human mpl ligand molecules that have been truncated (as compared to the 332 amino acid protein encoded by the cDNA in humans) so that they extend at least 151 amino acids, starting from amino acid position 22 in FIG. 1, 15 retain biological activity *in vivo*. FIG. 2 (SEQ. ID NOS.: 3 and 4) shows one example of a truncated mpl ligand molecule which, in mature form, has 174 amino acids and has biological activity. In FIG. 2, the 174 amino acid long protein is shown attached to a 21 amino acid N-terminal leader sequence. This molecule was 20 used to create some of the mpl ligand analogs in the examples section below. Other analogs are based on amino acids 1-199, 1-191, and 1-183 of FIG. 1. It is 25 also possible to remove up to the first six amino acids at the N-terminus of the mature human sequence mpl ligand protein and retain biological activity. Therefore, it appears that biological activity is 30 retained within amino acids 7 to 151 (inclusive) of the mature amino acid sequence of human mpl ligand shown in FIG. 1.

In general, many cell surface and secretory proteins produced by eukaryotic cells are modified with one or more oligosaccharide groups. This modification, referred to as glycosylation, can dramatically affect 35 the physical properties of proteins and can also be important in protein stability, secretion, and

A-337B

- 3 -

subcellular localization. Proper glycosylation can be essential for biological activity. In fact, some genes from eukaryotic organisms, when expressed in bacteria (e.g., *E. coli*) which lack cellular processes for 5 glycosylating proteins, yield proteins that are recovered with little or no activity by virtue of their lack of glycosylation.

Glycosylation occurs at specific locations or sites along the polypeptide backbone and is usually of 10 two types: O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides (chains) are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except 15 proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is 20 N-acetyleneuraminic acid (hereafter referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycoprotein.

25 As used herein glycosylation "sites" are amino acid residues that are structurally able to link to glycosyl residues, although such sites may or may not be actually linked to a glycosyl residue. As noted above, O-linked sites are either Ser or Thr residues, whereas 30 N-linked sites are either Asn-X-Ser or Asn-X-Thr, where X is defined as any amino acid other than Pro (preferably one of the 19 naturally-occurring amino acids, excluding Pro). Whether a given site is glycosylated with a glycosyl chain is determined by the 35 host cell in which the molecule is expressed, the amino acids neighboring the site, and other factors.

A-337B

- 4 -

As used herein, the number of "chains" attached to a given mpl ligand analog will be the average number of carbohydrate (i.e., glycosyl) chains attached to a given mpl ligand molecule expressed by a 5 particular host cell. Notably, the glycosylation sites for natural and corresponding recombinant mpl ligand will generally be the same, whereas the number of chains will possibly vary depending upon whether the particular host cell used for recombinant expression attaches 10 glycosyl chains to the same sites or not, as compared to the natural source. Herein, whenever a comparison is made between recombinant and natural mpl ligand analogs, the same number of amino acids will be compared, regardless of whether the natural source actually 15 produces an mpl ligand molecule having that length. Thus, "natural" refers to the sequence employed in a particular species (such as human) rather than the length of the molecule actually expressed in such natural source.

20 Naturally occurring mpl ligand is a glycosylated molecule. The glycosylation pattern of natural mpl ligand is related to two key domains that have been found in mpl ligand. The sequence of the first approximately 151 amino acids of mature human mpl 25 ligand, corresponding to an active portion of the molecule, bears notable homology to erythropoietin (EPO), a cytokine capable of stimulating production of erythrocytes, and is referred to as the "EPO-like" domain of human mpl ligand. The remaining amino acids 30 of the mature protein make up a so-called "N-linked carbohydrate" domain, since they include most if not all of the natural sites for N-linked glycosylation. In human mpl ligand, there are six N-linked glycosylation sites all contained in the N-linked glycosylation 35 domain. Both domains contain O-linked glycosylation sites. There are an estimated 12-14 O-linked

A-337B

- 5 -

glycosylation chains in the molecule. Experimental evidence with human mpl ligand DNA expressed recombinantly in CHO cells reveals that in the EPO-like domain at least two O-linked sites are glycosylated, at 5 positions 1 (Ser) and 37 (Thr).

Glycoproteins such as mpl ligand can be separated into different charged forms using techniques such as isoelectric focusing (IEF). For example, several parties have reported IEF studies of crude and partially 10 purified erythropoietin preparations (Lukowsky et al., *J. Biochem.* 50:909 (1972); Shelton et al., *Biochem. Med.* 12:45 (1975); Fuhr et al., *Biochem. Biophys. Res. Comm.* 98:930 (1981)).

In spite of the above information on 15 glycosylation of mpl ligand molecules, there remains a need to obtain mpl ligand molecules having a different glycosylation pattern and which retain or have improved biological activity.

Accordingly, it is an object of the present 20 invention to provide novel glycosylated mpl ligand molecules, referred to as mpl ligand analogs. It is a further object of this invention to provide pharmaceutical compositions containing such molecules and methods of treating conditions treatable by mpl 25 ligand with the mpl ligand analogs of this invention.

#### Summary of the Invention

In one embodiment, the subject invention relates to analogs of mpl ligand comprising an amino 30 acid sequence which includes at least one added, at least one deleted, and/or a combination of at least one added and deleted, site for glycosylation as compared to the corresponding natural sequence mpl ligand. The added or deleted site(s) for glycosylation may result in 35 a greater or lesser number of carbohydrate chains, and higher or lower sialic acid content, than corresponding

A-337B

- 6 -

natural sequence mpl ligand, particularly human mpl ligand. For example, one type of analog could involve deleting one or more N- or O-linked sites, and addition of one or more N- or O-linked sites at the same or 5 another position.

In another aspect of the above embodiment, the subject invention relates to mpl ligand analogs comprising amino acid sequences which involve replacement of one or more N- or O-linked glycosylation 10 sites with one or more non-naturally occurring sites. Thus, an N-linked site may be replaced with a different N-linked site; an N-linked site may be replaced with an O-linked site; an O-linked site may be replaced with a different O-linked site; and an O-linked site may be 15 replaced with an N-linked site.

Combinations of any of the above changes are further encompassed within this invention.

The invention further encompasses DNA sequences encoding such mpl ligand analogs, and recombinant plasmids 20 and host cells for analog expression.

In all of the above cases, the change in glycosylation site results in a change in the number, amount, location or type (N- vs. O-) of glycosyl chains in the resulting mpl ligand analog and retains a 25 biological activity of mpl ligand, i.e., the analog can still activate the mpl receptor. Activation of the mpl receptor means that megakaryocytopoiesis is enhanced thereby resulting in an increase in platelets *in vivo*.

30 Brief Description of the Drawings

FIG. 1 shows the DNA and amino acid sequence of native human mpl ligand including a signal peptide (amino acids -21 to -1) and the mature amino acid sequence (1-332).

35 FIG. 2 shows the DNA and amino acid sequence of mpl ligand corresponding to amino acids 1-174 of the

A-337B

- 7 -

human mature mpl ligand sequence attached to a 21 amino acid long signal peptide. The sequences flanking the coding regions have introduced XbaI and SalI cloning sites at the 5' and 3' ends respectively.

5 FIG. 3 shows a Western blot with *E. coli* and CHO expressed mpl ligand. MK stands for Met-Lys, which is added to the N-terminus of mpl ligand for expression in *E. coli*, and may be cleaved off using a dipeptidase, such as cathepsin C. A molecule in which MK has been  
10 removed is referred to as desMK. Treatment with the glycosidases neuraminidase and O-glycanase is indicated.

15 FIG. 4 shows *in vivo* activity of *E. coli* and CHO expressed mpl ligand in normal mice, in terms of platelet counts. The data indicates that glycosylated  
20 mpl ligand (CHO material) has superior activity than non-glycosylated (*E. coli*) material. This may be a result of increased half-life for the glycosylated material. For example, CHO 332 stands for human mpl ligand amino acids 1-332 (FIG. 1) expressed in CHO cells.

25 FIG. 5 shows a Western blot analysis of COS cell supernatants of recombinant human mpl ligand and analogs 4, 6, 7, 9, 10, and 11. The construction of the  
30 analogs is described in Example 4. Analogs 4, 7, 10 have at least one additional carbohydrate chain as evidenced by slower gel mobility. The analog numbers correspond to analog numbers provided in Table 1 (e.g., 11 corresponds to analog N11). The control is N1 in Table 1.

35 FIG. 6 shows a Western blot analysis of COS cell supernatants of recombinant human mpl ligand and analogs 4, 5, 13, 14, and 15. The construction of the analogs is described in Example 4. Analogs 4, 13, 14, and 15 have at least one additional carbohydrate chain as evidenced by slower gel mobility.

FIG. 7 shows a Western blot analysis of COS

A-337B

- 8 -

cell supernatants of human mpl ligand and indicated mpl ligand analogs after treatment with N-glycanase. The results indicate that the analogs have differential glycosylation patterns.

5 FIG. 8 shows the results of a human megakaryocyte growth bioassay with mpl ligand analogs. Panels A and D are the positive and negative controls respectively. The well pictured in panel A received 37.5 pg of wild type (i.e., natural sequence) mpl ligand  
10 1-174 COS-1 conditioned medium and shows substantial megakaryocyte growth. Panel D received 1.5 ul of COS-1 mock conditioned medium and shows no growth. Panels B and C are mpl ligand 1-174 analogs 7 and 10 respectively. Panel B received 9.0 pg of mpl ligand  
15 COS-1 conditioned medium while panel C received 27 pg and both show excellent megakaryocyte growth.

FIG. 9 shows a Western blot analysis of CHO mpl ligand 1-174 and analogs N4 and N15 (see Table 1). Slower gel mobility demonstrates that analog N4 (4B) has 20 one additional oligosaccharide while analog N15 (15-8) has two additional oligosaccharides.

FIG. 10 shows a Western blot of CHO cell-produced mpl ligand analogs with and without treatment with N-glycanase as indicated. Slower gel mobility 25 after treatment with N-glycanase demonstrates the presence of N-linked oligosaccharide.

FIG. 11 shows platelet counts from mice treated with various forms of mpl ligand at various doses. The data demonstrate that increased amounts of 30 N- and/or O-linked carbohydrate result in increased in vivo activity.

FIG. 12 shows a Western blot analysis of COS-produced mpl ligand 1-174, along with analogs N10, N15, N33, N39, N31, N35, and N40. The number of added N-35 linked glycosyl sites is also indicated. The figure shows that increasing the number of N-linked sites

A-337B

- 9 -

reduces the mobility of mpl ligand due to increasing amounts of N-linked carbohydrate.

FIG. 13 shows a Western blot analysis of COS-produced mpl ligand 1-174, along with analogs N15, N29, 5 N30, and N38. The number of N-linked glycosyl chains is also indicated.

#### Detailed Description of the Invention

The subject invention provides mpl ligands 10 with different glycosylation sites as compared to natural mpl ligand having a corresponding sequence. Preferably, the resulting molecules are those having additional glycosylation sites that are occupied by glycosyl chains upon expression in a mammalian cell 15 (such as COS, CHO, and human cells).

In a first embodiment, the subject invention relates to analogs of mpl ligand comprising an amino acid sequence which includes at least one added, at least one deleted, and/or at least one added and 20 deleted, site for glycosylation as compared to corresponding natural sequence mpl ligand. The added or deleted site(s) for glycosylation may result in a greater or lesser number of carbohydrate chains, and higher or lower sialic acid content, than corresponding 25 natural sequence mpl ligand, particularly human mpl ligand. A combination of a deletion of one site and addition of another site would result in no net change in the number of sites, but rather, a change in location and/or type of site. Such combined change analogs are 30 also encompassed within this invention.

In another aspect of the above embodiment, the subject invention relates to mpl ligand analogs comprising amino acid sequences which include replacement of one or more N- or O-linked glycosylation 35 sites with one or more non-naturally occurring sites. Thus, an N-linked site may be replaced with a different

A-337B

- 10 -

N-linked site; an N-linked site may be replaced with an O-linked site; an O-linked site may be replaced with a different O-linked site; and/or an O-linked site may be replaced with an N-linked site. Replacement of one site 5 with another site in essentially the same location may have the result of increasing the glycosylation efficiency at that site, or other effects. For example, evidence is provided herein that a Thr residue instead of a Ser residue may increase the glycosylation 10 efficiency at O-linked sites.

The term "mpl ligand", as used herein, includes naturally occurring mpl ligand, truncations of naturally occurring mpl ligand as well as non-naturally occurring polypeptides having an amino acid sequence and 15 glycosylation sufficiently duplicative of that of naturally occurring mpl ligand to allow possession of a biological activity of specifically stimulating growth, development and/or production of megakaryocytes and/or platelets. Mpl ligand analogs based on at least amino 20 acids 7-151 up to amino acids 1-332 of FIG. 1 are preferred.

In a preferred embodiment, mpl ligand is the product of the expression of an exogenous DNA sequence that has been transfected into a eukaryotic host cell; 25 that is, in a preferred embodiment the mpl ligand is "recombinant mpl ligand". The preferred eukaryotic host is mammalian, particularly preferably CHO cells. Recombinant mpl ligand is advantageously produced according to the procedures described herein and in the 30 publications cited herein regarding cloning and expression of mpl ligand.

Some additional preferred mpl ligand molecules have the following amino acid sequences, based on FIG. 1 herein:

	mpl ligand 1-332	amino acids 1-332 of FIG. 1
	mpl ligand 1-199	amino acids 1-199 of FIG. 1
	mpl ligand 1-191	amino acids 1-191 of FIG. 1
5	mpl ligand 1-183	amino acids 1-183 of FIG. 1
	mpl ligand 1-174	amino acids 1-174 of FIG. 1
	mpl ligand 1-163	amino acids 1-163 of FIG. 1
	mpl ligand 1-153	amino acids 1-153 of FIG. 1
	mpl ligand 1-152	amino acids 1-152 of FIG. 1
10	mpl ligand 1-151	amino acids 1-151 of FIG. 1
	mpl ligand 7-332	amino acids 7-332 of FIG. 1
	mpl ligand 7-199	amino acids 7-199 of FIG. 1
	mpl ligand 7-191	amino acids 7-191 of FIG. 1
	mpl ligand 7-183	amino acids 7-183 of FIG. 1
15	mpl ligand 7-174	amino acids 7-174 of FIG. 1
	mpl ligand 7-163	amino acids 7-163 of FIG. 1
	mpl ligand 7-153	amino acids 7-153 of FIG. 1
	mpl ligand 7-152	amino acids 7-152 of FIG. 1
	mpl ligand 7-151	amino acids 7-151 of FIG. 1

20

It should be noted, for example, that mpl ligand 1-183, 1-191, 7-183, and 7-191 encompass one or two additional naturally-occurring glycosylation sites on the C-terminus thereof, as compared to shorter sequences. In each of the above cases, Met-Lys may further be included in the N-terminus thereof.

The *in vitro* specific activities referred to herein are measurements of relative *in vitro* specific activities and are not measurements of absolute *in vitro* specific activities. For the purposes of this application, the specific activities are used only to compare relative activities of mpl ligand analogs that have been assayed using the same assay, using the same conditions including the same internal standard, and having the same analysis of the data used to calculate specific activity, etc.

A-337B

- 12 -

As used herein the phrases "analog of mpl ligand" or "mpl ligand analog" refer to mpl ligand with one or more changes in the amino acid sequence of mpl ligand which result in a change in the type (N- or O-linked, which may affect the amount of carbohydrate attached), number, or location of sites for carbohydrate attachment. In a preferred embodiment, the change in glycosylation site(s) results in a change in the number of glycosyl chains attached to the mpl ligand molecule.

5 In a particularly preferred embodiment, the change in glycosylation site(s) adds at least one (generally 1-6, preferably 1-5, particularly preferably 2-4) glycosyl chains, and most preferably the chain(s) is(are) added via N-linkage. In another particularly preferred

10 embodiment, the mpl ligand analog retains at least equivalent biological activity in vivo as compared to natural sequence mpl ligand (e.g., human mpl ligand) and may possess substantially higher activity in vivo, as measured in assays for biological activity. Such assays

15 include those that detect megakaryocyte or platelet production.

20

To prepare such analogs of mpl ligand, preferably they are generated by site-directed mutagenesis resulting in additions, deletions, or 25 substitutions of amino acid residues that add, eliminate or alter sites that are available for glycosylation. By "altered" is meant that a site has been deleted while another has been added at the same or another location as the deleted site. However, as is appreciated by 30 those skilled in the art, other methods could result in a gene encoding the same amino acid sequence, and such methods are encompassed herein. The resulting analogs may have fewer or more (preferably more) attached carbohydrate chains than natural human/recombinant mpl 35 ligand.

Addition of one or more carbohydrate (i.e.,

A-337B

- 13 -

glycosyl) chains to mpl ligand is one important object of this invention. Mpl ligand analogs having more carbohydrate chains than those found in the corresponding naturally-occurring amino acid sequence 5 (e.g., 1-332 or 1-174, etc.) are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation in a way that would substantially reduce biological activity. As used herein the "naturally-occurring" mpl ligand refers to an 10 amino acid sequence having the corresponding number of amino acids as the relevant analog, even if the particular length of mpl ligand species is not actually expressed in the native species. Advantageously, the analog of mpl ligand has up to 6 additional sites for 15 N-glycosylation or O-glycosylation, resulting in the addition of from 1 up to 6 additional N-linked or O-linked carbohydrate chains (or a combination thereof).

For example, a Pro at position 30 is replaced by an Asn and a Val at position 32 is replaced by a Thr 20 to give the sequence Asn-Glu-Thr, which serves as a new site for N-glycosylation (analog N4 below; see Table 1).

Analogs may also be constructed which have two or more additional N-linked chains by combining mutations; for example, analogs N4 and N10 described in 25 Table 1 may be combined to yield an analog with two additional sites for carbohydrate addition (i.e., analog N15 in Table 1). In a like manner analogs with three or more added chains can be constructed. As will be appreciated by those skilled in the art, the subject 30 invention includes many other analogs of mpl ligand having different sites for glycosylation (in terms of number, type or location of site). The mpl ligand analogs of this invention are in all cases particularly preferably based on mpl ligand having a human amino acid 35 sequence (see FIGs. 1 and 2); however, analogs based on mpl ligand sequences from other species (e.g., dog, pig,

A-337B

- 14 -

monkey, mouse or rat) are also contemplated herein.

Insertions of amino acids to create glycosylation sites are also contemplated. For example, a Glu at position 57 is replaced by a Thr and Asn is inserted immediately after Met at position 55 as follows:

-Gln-Met-Glu-Glu-Thr-  
54 55 56 57 58  
→

10 -Gln-Met-Asn-Glu-Thr-Thr-  
54 55 55' 56 57 58

This adds a new glycosylation site (amino acids 55', 56, and 57). See analog N23 below.

Also included within the analogs of this invention are analogs which have one or more amino acids extending from the carboxy terminal end of mpl ligand wherein the carboxy terminal extension provides at least one additional carbohydrate site. The carboxy terminus of mpl ligand will vary depending upon the particular form of mpl ligand used (e.g., mpl ligand 1-332 amino acids, or mpl ligand 1-163 amino acids). An additional carbohydrate site may be added to the carboxy terminus of an mpl ligand species by adding amino acids to the carboxy terminus, such amino acids containing one or more N- or O-linked glycosylation sites.

Tables 1 and 6 list some exemplary mpl ligand analogs which have additional sites for N-linked carbohydrate chains. The analogs have the sequence Asn-X-Ser or Asn-X-Thr included at various positions in the human mpl ligand polypeptide chain based on the human amino acid sequences to create N-linked sites. Tables 4 and 7 list those analogs which add at least one additional N-linked carbohydrate chain, as evidenced by the migration of the glycoproteins on SDS gels (see, Example 6 and FIGs. 3, 5, 6, 7, 9, 10, 12, and 13). Note that these Tables also include some truncated

A-337B

- 15 -

species that are not "analog" as defined herein (i.e., N1, N16, N17, and N31). These are listed in the Tables to show how various truncated species were prepared.

Also encompassed by the present invention are 5 DNA sequences encoding the mpl ligand analogs disclosed herein, preferably those encoding analogs having additional sites for N-linked chains. Procedures used to introduce changes into the mpl ligand DNA sequence for the purpose of creating, deleting and/or altering 10 attachment sites for carbohydrates are disclosed in Examples 4 and 14.

These mpl ligand analogs can be the product of expression of an exogenous DNA sequence, i.e., produced through recombinant DNA technology, they can be 15 chemically synthesized products or they may be produced by combined methods. An exogenous DNA sequence comprises cDNA, genomic DNA or chemically synthesized DNA encoding an mpl ligand analog. Recombinant DNA plasmids and eukaryotic host cells useful for the 20 expression of such analogs are also provided. Expression vectors include any vector which is capable of expressing cloned DNA sequences in a eukaryotic host cell, particularly those vectors used for expression in COS and CHO cells. Examples of such vectors include 25 plasmids pDSR $\alpha$  and pDSR $\alpha$ 2, see Mol. Cell. Biol. 8:466-472 (1988); WO 91/13160 (1991); and WO 90/14363 (1990). The cultivation of COS and CHO host cells expressing mpl ligand analogs was carried out using standard procedures known to those skilled in the art.

30 Changing the number, type, location, or amount of carbohydrate chains attached to mpl ligand may confer advantageous properties such as increased solubility, greater resistance to proteolysis, reduced immunogenicity, increased serum half-life, and increased 35 or altered biological activity.

Conditioned media from COS cells expressing

A-337B

- 16 -

mpl ligand analogs N2 - N15 (N1 is human mpl ligand 1-174; see FIG. 2) were analyzed for *in vitro* biological activity and the results shown in Table 4.

5 Conditioned media from COS cells expressing  
mpl ligand analogs/truncations N15-N40 were analyzed for  
*in vitro* biological activity and the results are shown  
in Table 7.

10 In vivo biological activity results for  
various forms are presented in FIG. 11 (see Example 13).

15 Another embodiment of the invention relates to  
mammalian (e.g., Chinese Hamster Ovary, CHO) host cells  
which preferentially synthesize mpl ligand or analogs of  
mpl ligand having greater than a specific number of  
sialic acids per molecule, e.g. greater than that found  
in mpl ligand 1-332, 1-199, 1-191, 1-183, 1-174, 1-163,  
1-153, 1-152, or 1-151 produced naturally or  
recombinantly in a eukaryotic cell. In vitro activities  
of analogs N4 and N15, along with full-length and  
various truncated species expressed in CHO cells are  
20 shown in Table 5.

25 The sialic acid content of the mpl ligand  
molecule may affect its *in vivo* biological activity.  
For example, tetraantennary (four-branched) N-linked  
oligosaccharides most commonly provide four possible  
sites for sialic acid attachment while bi- and  
triantennary oligosaccharides, which can substitute for  
the tetraantennary form at asparagine-linked sites,  
commonly have at most only two or three sialic acids  
attached. O-linked oligosaccharides commonly provide  
30 two sites for sialic acid attachment. Thus, mpl ligand  
molecules with N-linked carbohydrate substituted for  
O-linked carbohydrate can accommodate two additional  
sialic acids per chain provided the N-linked  
oligosaccharides are tetraantennary. Mammalian cell  
35 cultures are screened for those cells that  
preferentially add tetraantennary chains to recombinant

A-337B

- 17 -

mpl ligand, thereby maximizing the number of sites for sialic acid attachment.

Dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary (CHO) cells are a commonly used 5 host cell for the production of recombinant glycoproteins including recombinant mpl ligand.

10 Compositions comprising a therapeutically effective amount of an mpl ligand analog in accordance with this together with a suitable diluent, adjuvant and/or carrier useful in mpl ligand therapy are further encompassed by this invention. A "therapeutically effective amount" as used herein refers to that amount which provides therapeutic effect for a given condition and administration regimen.

15 The present compositions can be systemically administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may 20 be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art. The specific route chosen 25 will depend upon the condition being treated. The administration of mpl ligand or mpl ligand analogs is preferably done as part of a formulation containing a suitable carrier, such as human serum albumin, a suitable diluent, such as a buffered saline solution, 30 and/or a suitable adjuvant. The required dosage will be in amounts sufficient to raise the platelet levels of patients and will vary depending upon the severity of the condition being treated, the method of administration used and the like.

35 The conditions to be treated by the methods and compositions of the present invention are generally

A-337B

- 18 -

those which involve an existing megakaryocyte/platelet deficiency or an expected megakaryocyte/platelet deficiency in the future (e.g., because of planned surgery). Such conditions will usually be the result of 5 a deficiency (temporary or permanent) of active mpl ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available for treating thrombocytopenia.

10 Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy, bone marrow transplants, and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions.

15 Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic tumors which result in thrombocytopenia, systemic lupus erythematosus,

20 splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound 25 healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery or future thrombocytopenia-inducing therapy, an mpl ligand analog 30 of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, an mpl ligand analog could be administered along with blood or purified platelets.

35 The dosage regimen involved in a method for treating the above-described conditions will be

A-337B

- 19 -

determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of

5 administration and other clinical factors. Generally, the daily regimen should be in the range of 0.01-1000 micrograms of mpl ligand analog per kilogram of body weight, preferably 0.1-10 micrograms per kilogram of body weight.

10 The therapeutic methods, compositions and polypeptides of the present invention may also be employed, alone or in combination with other cytokines, soluble mpl (i.e., mpl ligand) receptor, hematopoietic factors, interleukins, growth factors or antibodies in

15 the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that an mpl ligand analog molecule will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of

20 hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed

25 with mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO,

30 interferon-alpha (IFN-alpha), IFN-beta, or IFN-gamma. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into

35 platelets once the megakaryocytes have reached mature form. Thus, administration of mpl ligand analog (to

A-337B

- 20 -

enhance the number of mature megakaryocytes) followed by administration of the soluble mpl receptor (to inactivate the analog and allow the mature megakaryocytes to produce platelets) is expected to be a 5 particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

10 Additional modifications of the analogs of this invention may also be carried out, e.g., to increase activity, stability, half-life, etc. For example, pegylation (poly- or mono-) could be added to the mpl ligand analog via amino groups on the protein or 15 via the carbohydrate groups. Also, fatty acids or other polymers could be attached to the protein or carbohydrate groups.

20 The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. The mpl ligand standard used in the bioassays employed in the Examples is a recombinant mpl ligand standard that was expressed in *E. coli*, refolded into an active conformation and purified. Thus, only relative specific activities are 25 being measured.

## EXAMPLE 1

Construction of Mpl Ligand 1-174

30 Human mpl ligand gene encoding amino acids 1-174 (starting with S-P-A-P-P-A...) of FIG. 2 was generated from a human fetal liver cDNA library (Bartley et al, Cell 77: 1117-1124 (1994) by polymerase chain 35 reaction (PCR). The 5' PCR primer encoded the amino terminus of human mpl ligand, an XbaI site, and an

A-337B

- 21 -

optimized Kozak sequence. The 3' primer contained a termination codon and a SalI restriction site. The amplified DNA fragment was digested with XbaI and SalI then ligated to XbaI and SalI cut pDSR $\alpha$ 2. The resultant 5 plasmid, pDSR $\alpha$ 2 mpl ligand 1-174 was used for mammalian cell expression. The sequence of the resulting gene (including the signal peptide) is shown in FIG. 2.

Plasmid DNA containing mpl ligand 1-174 was digested with XbaI and SalI restriction enzymes, the 10 resulting DNA fragments were subjected to agarose gel electrophoresis, and the 605 nt mpl ligand 1-174 DNA fragment was isolated from the gel using a GeneClean™ kit and procedures supplied by the manufacturer (BIO 101, Inc.). Plasmid pDSR $\alpha$ 2 as described in WO 90/14363 15 (1990) was also digested with XbaI and SalI restriction enzymes and the vector fragment was recovered. Ligation of the two fragments results in pDSR $\alpha$ 2 (mpl ligand 1-174).

20

## EXAMPLE 2

Expression of Mol Ligand 1-174 in CHO Cells and Purification

25 Dihydrofolate reductase deficient (DHFR $^{-}$ ) Chinese Hamster Ovary (CHO) cells were transfected with pDSR $\alpha$ 2-mpl ligand 1-174. A 100 mm tissue culture dish was plated with  $1 \times 10^6$  CHO DHFR $^{-}$  cells grown in CHO D $^{-}$  medium (DMEM, 10% Fetal bovine serum, 1% penicillin/ 30 streptomycin/glutamine, 1% nonessential amino acids (Gibco) and 1% HT supplement (Gibco)) the day before transfection. Four transfections were performed. For each transfection, plasmid DNA (50 ug) was linearized by digesting with Pvu I and Buffer H 35 (Boehringer Mannheim). A DNA precipitate was then formed and added to the plates dropwise as per the

A-337B

- 22 -

Mammalian Cell Transfection Kit (Speciality Media). After 24 hours in a tissue culture incubator the medium was replaced with fresh CHO D- medium. Twenty four hours later the cells were split into 96 well tissue culture 5 plates with 100 ul of CHO select medium (D-MEM, 5% dialyzed fetal Bovine serum, 1% penicillin/streptomycin/glutamine, 1% nonessential amino acids (Gibco)) per well and transformants were selected. Medium was changed weekly until colonies 10 appeared. After two weeks, mpl ligand expression was screened for using the 32D cell proliferation assay described below (see Example 9). Those clones expressing in excess of  $1 \times 10^5$  units/ml were expanded and frozen in cryogenic storage. One clone was expanded for 15 roller bottle production and approximately 8 liters of conditioned medium was produced.

Plasmid pDSR $\alpha$ 2 containing mpl ligand 1-174 cDNA was transfected into DHFR-deficient CHO cells as explained above. Two liters of serum-free CHO cell 20 conditioned medium (50% D-MEM, 50% HAMS-F12, 1% penicillin/ streptomycin/glutamine, 1% nonessential amino acids(Gibco)) from roller bottles seeded with CHO cells expressing mpl ligand 1-174 was concentrated 15 fold using a 2L Amicon Model 2000 stirred cell and a 25 10,000 dalton molecular weight cut-off membrane (YM10, Amicon). Forty-five milliliters of concentrated conditioned medium was then loaded directly onto a 4 ml hu-MPL-X affinity column at a flow rate of 0.4 ml/min using a Pharmacia FPLC. The affinity column was 30 constructed by coupling 1.5-2.5 milligrams of Mpl-X (the soluble extra-cellular domain of the mpl receptor) per milliliter of Pharmacia CNBr activated Sepharose resin as recommended by the manufacturer. After loading, the column was washed with 16 ml of phosphate buffered 35 saline (PBS; 10 mM Na $\cdot$ PO<sub>4</sub> pH 6.8/150 mM NaCl) and then 24 ml of 10 mM Tris, pH 8.0 / 1M NaCl. Mpl ligand

A-337B

- 23 -

(1-174) was eluted with 40 ml of 20 mM CAPS (3-[Cyclohexylamino]-1 propanesulfonic acid) pH 10.5 / 1M NaCl / 5 mM CHAPS(3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) in 6 ml fractions.

5 The second fraction yielded a single band on a 14% SDS gel. This material was concentrated and buffer exchanged against a saline solution of 0.9% NaCl and was biologically active *in vitro* and *in vivo*. Other forms of CHO cell expressed mpl ligand were purified in a

10 similar manner.

## EXAMPLE 3

In vivo Biological Activity of Recombinant Human Mpl  
15 Ligand

Platelet counts from mice treated with various forms of mpl ligand were measured. CHO-derived mpl ligand 1-332, 1-174, 1-163, and 1-153 were produced and

20 purified by Mpl-receptor affinity chromatography.

*E. coli*-derived Met-Lys-mpl ligand 1-332, Met-Lys-mpl ligand 1-174, Met-Lys-mpl ligand 1-163 and Met-Lys-mpl ligand 1-153 were produced and purified by conventional chromatography.

25 FIG. 4 shows platelet counts from mice treated with various forms of CHO cell-derived (solid lines) or *E. coli*-derived (dashed lines) recombinant human mpl ligand. Normal, female Balb/c mice were injected subcutaneously with the indicated concentration 30 of mpl ligand for 5 consecutive days. Test bleeds from a small lateral cut in a tail vein were collected 24 hours after the last injection. Blood cell analyses were performed with a Sysmex electronic blood cell analyzer (Baxter Diagnostics, Inc. Irvine, CA). Data 35 are represented as the mean of determinations of 4

A-337B

- 24 -

animals, +/- standard error of the mean. Other blood cell parameters such as total white blood cell counts or red blood cell counts were not affected by these treatments (data not shown).

5 The results indicate that CHO cell expressed forms of mpl ligand have an increased *in vivo* activity relative to the same forms of mpl ligand produced in *E. coli*. As described in Example 6, the CHO cell expressed forms of mpl ligand all contain N and/or O-linked 10 carbohydrate and the *E. coli* expressed mpl ligand forms do not. This indicates that the carbohydrate enhances the *in vivo* activity of mpl ligand. The increased *in vivo* activity conferred by the carbohydrate may be a 15 result of increased circulatory half life, increased stability or a combination of both.

#### EXAMPLE 4

##### Construction of Mol Ligand Analogs N2-N15

20

Procedures for generating additional glycosylation sites for mpl ligand are described below.

25 The following oligonucleotide primers were synthesized for use in *in vitro* mutagenesis to prepare analogs N2-N14 (see Table 1 for the structures of these analogs):

	N2 - CCCATGTCAATCACAGCAGACT	SEQ ID NO.: 5
	N3 - CTTCACAGCAACCTGAGCCAGT	SEQ ID NO.: 6
30	N4 - CAGTGCAACGAGACCCACCCCTTG	SEQ ID NO.: 7
	N5 - GCCTACAAATGTCACGCTGCCGTGCT	SEQ ID NO.: 8
	N6 - CCCACTTGTAACTCATCCCTC	SEQ ID NO.: 9
	N7 - CAACTGAAACGCCACTTGTCTCTCA	SEQ ID NO.: 10
	N8 - ACTTGTCTCAACTCCACCCCTGGGGGA	SEQ ID NO.: 11
35	N9 - CTCCTGGGAAACCTTCTGGAA	SEQ ID NO.: 12
	N10 - GACCACAAATCACACCGATCCCAAT	SEQ ID NO.: 13

A-337B

- 25 -

N11 - ACCCTTGTCTACAAATGTCACGCTGCCTGCT	SEQ ID NO.: 14
N12 - TCTCTCAAACCTCACGGGGAGCTT	SEQ ID NO.: 15
N13 - TGGAAAATCAGACGGAGGAGAC	SEQ ID NO.: 16
N14 - TGGAGGAGAACAGACACAGGACAT	SEQ ID NO.: 17

5

To construct m13mp18 mpl ligand 1-174, the gene of FIG. 2 was introduced into XbaI and SalI restriction enzyme digested m13mp18 DNA. Single stranded DNA was recovered from supernatants of *E. coli* strain RZ1032 infected by m13mp18(mpl ligand 1-174) as described by Kunkel et al., *Methods in Enzymol.* 154:367 (1987) and Messing, *Methods in Enzymol.* 101:20 (1983). For *in vitro* mutagenesis approximately 0.5 µg of single-stranded DNA and 0.125 pmole of one of the synthetic primers described above were mixed with 6 µl of buffer (250 mM Tris pH 7.8, 50 mM MgCl<sub>2</sub>, 50 mM dithiothreitol and 1% Bovine serum albumin (BSA-Pharmacia)). The primers were previously kinased with ATP and T4 polynucleotide kinase prior to addition. For annealing of the primer to the template, the reaction volume was adjusted to 10 µl with water, the mixture was heated to 65°C for 5 minutes and then allowed to cool to room temperature. For the elongation reaction 2.5 µl of each of dTTP, dATP, dGTP and dCTP and 1 ml ATP (all at 10 µM) were added, followed by 1 µl (1 unit) of *E. coli* DNA polymerase (Klenow fragment) and 1 µl (1 unit) of T4 DNA ligase. The mixture was then incubated overnight at 14°C and used to transform *E. coli* JM 109 (Yanisch-Perron et al. *Gene* 33, 103 (1985)) as described (Messing, *supra*).

To identify mutant clones by differential hybridization, plaques on nutrient agar were transferred to Gene Screen filters (New England Nuclear). The DNA was cross-linked to filters by irradiating them in a UV Stratalinker Model 1800 using the auto cross-link mode (Stratagene). They were then incubated for one hour in

A-337B

- 26 -

6x SSC (0.9M NaCl/0.09M Na-citrate) containing 1% SDS at 60°C. For the hybridization, the oligonucleotide primer above (8 pmoles) was end-labeled with T4 polynucleotide kinase and  $\gamma$  <sup>32</sup>P-labeled ATP and incubated with the filters overnight in 6x SSC, 0.5% SDS and 125 ug/ml herring sperm DNA. The hybridization temperatures were chosen according to estimates of oligonucleotide melting points. Generally the hybridization temperature was approximately 10°C less than the melting point. The next day, the filters were washed two times with 6X SSC/1% SDS at hybridization temperature followed by two washes with 6x SSC at hybridization temperature and subjected to autoradiography. If necessary, the filters were then washed with 6x SSC at increasing temperatures until little or no hybridization was detected to plaques having the wild-type mpl ligand cDNA sequence. Clones that gave positive hybridization signals under these conditions were identified and retransfected into JM109 to isolate a pure clone. Dideoxy chain termination sequence analysis indicated that the mutations were present.

Double stranded m13 mpl ligand 1-174 DNAs carrying the desired changes were recovered from JM109 transfected cells with QIAGEN kits (Chatsworth CA.) using methods supplied by the manufacturer. The DNAs were digested with XbaI and SalI and the 605 bp mpl ligand DNA fragments were isolated. pDSR $\alpha$ 2 was digested with XbaI and SalI. The vector fragment was isolated and ligated to the mpl ligand fragments above. Recombinant plasmids were identified by restriction analysis. The resulting plasmids (designated mpl ligand 1-174-NX where NX is the analog number) contain DNA encoding mpl ligand analogs having altered amino acid residues at the indicated positions. The resultant plasmids were then sequenced again to confirm the presence of the desired mutations.

A-337B

- 27 -

The analog N15 was constructed that had two additional N-linked glycosylation sites at positions 30 and 120. PDSR $\alpha$ 2 mpl ligand 174-N4 containing Asn30 and Thr32 mutations was digested with XbaI and PstI 5 restriction enzymes and the approximately 385 nt DNA fragment was isolated. PDSR $\alpha$ 2 mpl ligand 174-N10 containing Asn120 and Thr122 mutations was digested with PstI and SalI restriction enzymes and the approximately 220 nt DNA fragment was isolated. pDSR $\alpha$ 2 was digested 10 with XbaI and SalI. The vector fragment was isolated and ligated to the mpl ligand fragments above. This resulted in PDSR $\alpha$ 2 mpl ligand 174-N15 that contains Asn30, Thr32, Asn120 and Thr122 substitutions.

These general procedures were used to 15 construct the mpl ligand analogs shown in Table 1. The DNA sequence changes for each of the analogs are shown; otherwise the oligonucleotide primers used for mutagenesis had sequences complementary to those of human mpl ligand.

A-337B

- 28 -

TABLE 1

Species No.	Analog/ Substitution	Amino Acid Changes
N1	(1-174), Pro <sup>175</sup> →Gly <sup>332</sup> deleted	CCA→TGA (stop codon)
N2	Leu <sup>22</sup> →Asn <sup>22</sup>	CCT→AAT
N3	Arg <sup>25</sup> →Asn <sup>25</sup>	AGA→AAC
N4	Pro <sup>30</sup> , Val <sup>32</sup> →Asn <sup>30</sup> , Thr <sup>32</sup>	CCA, GTT →AAC, ACC
N5	Pro <sup>38</sup> , Leu <sup>40</sup> →Asn <sup>38</sup> , Thr <sup>40</sup>	CCT, CTG →AAT, ACG
N6	Leu <sup>86</sup> →Asn <sup>86</sup>	CTC→AAC
N7	Gly <sup>82</sup> , Pro <sup>83</sup> →Asn <sup>82</sup> , Ala <sup>83</sup>	GGA, CCC→AAC, GCC
N8	Ser <sup>87</sup> , Leu <sup>89</sup> →Asn <sup>87</sup> , Thr <sup>89</sup>	TCA, CTC→AAC, ACC
N9	Gln <sup>92</sup> →Asn <sup>92</sup>	CAG→AAC
N10	Ala <sup>120</sup> , Lys <sup>122</sup> →Asn <sup>120</sup> , Thr <sup>122</sup>	GCT, AAG→AAT, ACC
N11	Pro <sup>36</sup> , Pro <sup>38</sup> , Leu <sup>40</sup> → Ser <sup>36</sup> , Asn <sup>38</sup> , Thr <sup>40</sup>	CCT, CCT, CTG→ TCT, AAT, ACG
N12	Ser <sup>88</sup> Leu <sup>90</sup> →Asn <sup>88</sup> , Thr <sup>90</sup>	TCC, CTG→AAC, ACG
N13	Thr <sup>53</sup> , Met <sup>55</sup> →Asn <sup>53</sup> , Thr <sup>55</sup>	ACC, ATG→AAT, ACG
N14	Thr <sup>58</sup> , Ala <sup>60</sup> →Asn <sup>58</sup> , Thr <sup>60</sup>	ACC, GCA→AAC, ACA
N15	Pro <sup>30</sup> , Val <sup>32</sup> , Ala <sup>120</sup> , Lys <sup>122</sup> → Asn <sup>30</sup> , Thr <sup>32</sup> , Asn <sup>120</sup> , Thr <sup>122</sup>	CCA, GTT, GCT, AAG→ AAC, ACC, AAT, ACC

A-337B

- 29 -

Note: Analogs N2-N15 are synonymously referred to herein as analogs 2-15. Further, as used herein, for example, [Asn<sup>22</sup>] mpl ligand means that an asparagine has been substituted for the amino acid at position 22 in 5 the particular mpl ligand species being considered, which is preferably a human sequence having at least amino acids 7-151 of FIG. 1 (including the preferred human mpl ligand sequences set forth herein above). Thus, substitution of an asparagine residue for a 10 leucine residue at position 22 of mpl ligand 1-174 (human sequence) yields an mpl ligand analog that may be represented by [Asn<sup>22</sup>] mpl ligand 1-174.

Plasmids designated pDSRa2 1-174-NX (where NX 15 is the analog number) were constructed by inserting mpl ligand DNA into pDSRa2. The expression vector pDSRa2 is generally described in WO 90/14363 (1990). pDSRa2 mpl ligand 1-174-NX plasmids were made by digestion of pDSRa2 with XbaI and SalI. The vector fragment was 20 isolated and ligated to the approximately 605 bp fragments containing the desired sequences.

#### EXAMPLE 5

Expression of Mpl Ligand and Mpl Ligands N1-N15  
25 in COS Cells

cDNA clones of human mpl ligand and mpl ligand analogs described in Table 1 were transferred into COS-1 cells (ATCC No. CRL-1650) by electroporation. COS-1 30 cells were harvested from semi-confluent dishes, washed with medium (Dulbecco's modified essential medium containing 10% fetal bovine serum and 1% L-glutamine/penicillin/ streptomycin (Irvine Scientific)) and resuspended at 6 x 10<sup>6</sup> cells/ml. One 35 half ml of cells was transferred to a 0.2 cm electroporation cuvette (Bio-Rad) and electroporated

A-337B

- 30 -

with a BTX Electroporation System Electrocell Manipulator 600 at 650 uF and 130 volts on the low voltage setting with 50 µg of plasmid DNA encoding the mpl ligand analog. The electroporated cells were plated 5 on 100 mm tissue culture dish in 10 ml of medium. Twelve to twenty four hours after plating the medium was replaced with 10 ml of fresh medium. The conditioned medium was collected 3 to 5 days after electroporation.

10

## EXAMPLE 6

Characterization of Mpl Ligand and Mpl Ligands N1-N15

## A. Determination of Carbohydrate Addition

15

A volume of supernatant containing approximately 30-60 ng mpl ligand or mpl ligand analog from COS cells transfected with mpl ligand analog cDNAs as described in Example 5 was immunoprecipitated 20 overnight at room temperature with a rabbit anti-mpl ligand polyclonal antibody. In some cases where expression was low, a maximum volume of approximately 8-9 ml was used for immuno-precipitation. The antibody was raised to mpl ligand 1-163 that had been expressed 25 and purified from *E. coli*. Thirty µl of 1:1 Protein A-Sepharose in phosphate buffered saline (PBS) containing 0.1% sodium azide was added to the immunoprecipitate and allowed to incubate for one hour at room temperature. The samples were centrifuged, 30 washed with PBS and resuspended in SDS sample Buffer (0.125 M Tris-HCl pH 6.8/ 4% SDS/ 20% glycerol/ 10%  $\beta$ -mercaptoethanol/ 0.001% bromophenol blue). The samples were analyzed by 12% SDS-polyacrylamide gel 35 electrophoresis, transferred to nitrocellulose and subjected to Western analysis as described (Burnette et al., *Anal. Biochem.* 112:195-203 (1981); Elliott et al.,

A-337B

- 31 -

Gene 79:167-180 (1989)) using a mouse anti-mpl ligand monoclonal antibody raised to a synthetic mpl ligand peptide (e.g., corresponding to amino acid residues 47-62 of FIG. 1). The mpl ligand containing bands were 5 visualized using an ECL kit (Amersham).

FIG. 5 shows that COS cell supernatants from cells transfected with analogs N4, N7 and N10 DNA revealed increased size compared to human sequence mpl ligand 174 (N1). FIG. 6 shows that COS cell 10 supernatants from cells transfected with N13, N14 and N4 DNA also had increased size compared to human sequence mpl ligand. This increased size is indicative of an additional N-linked carbohydrate chain. N15 contains two additional N-linked glycosylation sites. FIG. 6 15 indicates that this analog has material with a size greater than analogs containing only 1 additional N-linked glycosylation. The sizes of the proteins were estimated from their mobility on SDS-PAGE relative to protein standards of known molecular weight. The 20 estimated sizes of the larger bands calculated from FIG. 6 are shown in Table 2. This result indicates that N15 contains 2 additional N-linked chains. Western blot analyses of other selected analogs are also shown in FIG. 6.

A-337B

- 32 -

TABLE 2

## N-Linked Carbohydrate Estimates

Mpl Ligand Analog (1-174)	Molecular Weight (Da)	Molecular Weight Shift(Da)	No. of Potential N-Linked Chains (Over Native) (⊕4 KDa/Site)
N1 (Native)	23500	0	0
N4	28700	5200	1
N7	27200	3700	1
N10	27200	3700	1
N13	26700	3200	1
N14	28700	5200	1
N15	33500	10000	2

5 An experiment was performed to show that the increased size of mpl ligand analogs is due to N-linked carbohydrate. COS cell conditioned medium containing mpl ligand was immunoprecipitated and washed with PBS as described above. To each tube was then added 10  $\mu$ l 0.5% SDS and each sample was boiled for 3 minutes. Then the following components were added: 10.8  $\mu$ l of 0.5M NaPO<sub>4</sub> pH 8.6, 5 ml of 7.5% nonidet P40 and 3  $\mu$ l of 250 unit/ml N-glycanase (Genzyme). N-glycanase treatment removes N-linked carbohydrate. Samples were incubated for 6 hours at 37°C. The reaction was stopped by the addition of SDS-PAGE sample buffer and then subjected to SDS-PAGE Western analysis (12% acrylamide) using an anti-mpl ligand monoclonal antibody and an anti-mouse ECL Western Detection Kit (Amersham) as described above. An analysis of N-linked chains using this method is shown in FIG. 7 for human mpl ligand and mpl ligand analogs. Following treatment with N-glycanase the mobility on Western blot for N4, N7 and N10 was reduced to that of N1. As expected, treatment of N1 with N-glycanase had no effect on mobility because N1 has no N-linked

A-337B

- 33 -

glycosylation sites. These results indicate that the increased size observed is due to addition of N-linked carbohydrate.

5 B. Analysis of O-linked carbohydrate on mpl ligand

To analyze the contribution of O-linked carbohydrate to human mpl ligand, various forms of the protein were purified from CHO cell conditioned media as 10 described above. Each form received +/- treatment with O-glycanase (Glycopeptide alpha-N-acetylgalactos-aminidase, Oxford GlycoSystems). O-glycanase removes O-linked carbohydrate from glycoproteins. The *E. coli* expressed version of each form was used as an 15 unglycosylated control. To resolve the difference in molecular weight contributed by O-linked carbohydrate, it was necessary to remove any N-linked carbohydrate first. Since the full length version, mpl ligand 1-332, contains N-linked carbohydrate, the CHO cell expressed 20 full length samples received N-glycanase (peptide-N4-(N-acetyl-beta-glucosaminy1) asparagine amidase) treatment as described above for COS cell expressed mpl ligand analogs, except that the N-glycanase treatment was an overnight incubation.

25 Before proceeding with the O-glycanase treatment on full length (1-332) mpl ligand, the pH range of the sample was adjusted to pH 6.0-pH 7.0 with 1/15 volume of 100 mM acetic acid, pH 2.2. One microgram of protein was denatured by boiling for 3 30 minutes in SDS and incubated at 37°C for 60 minutes with 1 U/ml neuraminidase (sialidase, from *Arthrobacter urefaciens*, Boehringer Mannheim) in 1 mM calcium acetate, pH 6.8 and 20 mM sodium phosphate, pH 6.8.

Subsequent treatment with O-glycanase was done 35 by adding 5 mU of enzyme in a final volume of 100 ul, followed by an overnight incubation at 37°C. Proteins

A-337B

- 34 -

(0.2 ug/lane) were separated by SDS-PAGE (15% acrylamide). Following transfer to 0.2 um nitrocellulose and overnight incubation with anti-mpl ligand polyclonal antibody the mpl ligand proteins were 5 visualized using an anti-rabbit ECL Western Detection Kit (Amersham).

FIG. 3 shows a Western blot of four different forms of human mpl ligand. Full length mpl ligand 1-332 is represented in lanes 1-3, mpl ligand 1-174 lanes 4-6, 10 mpl ligand 1-163 lanes 7-9, and mpl ligand 1-153 lanes 10-12. Treatment with neuraminidase and O-glycanase, shown in lanes 2,5,8, and 11, reduced the molecular weight to that of unglycosylated materials, lanes 3,6,9, and 12. In every case the mobility increased to that of 15 the unglycosylated version expressed in *E. coli*. These results indicate that the larger sized bands, in lanes 1,4,7, and 10 are due to O-linked carbohydrate. The molecular weight of each of the bands was estimated by comparing their mobilities to proteins of known 20 molecular weight.

As seen in Table 3 which shows estimated molecular weights of the different proteins, the apparent shift in mobility could account for as many as 14 O-linked carbohydrate chains (assuming 950 25 daltons/chain) for mpl ligand 1-332, 9 chains for mpl ligand 1-174, 4 chains for mpl ligand 1-163, and 2 chains for mpl ligand 1-153. The sample run in lane 2 is full length mpl ligand 1-332. It would appear that 30 this protein was degraded, possibly due to extended incubation in glycoenzymes at 37°C. Therefore, the *E. coli* expressed unglycosylated version in lane 3 was used to calculate the approximate molecular weight of O-linked carbohydrate added to CHO cell expressed mpl ligand 1-332.

35 These results are consistent with the presence of carbohydrate on all the CHO expressed forms of mpl

A-337B

- 35 -

ligand tested. The presence of O-linked carbohydrate was confirmed for CHO cell expressed mpl ligand 1-332, 1-174, and 1-163 by direct analysis of monosaccharide composition. Sialic acids, GalNAc and Gal were released from glycoproteins by acid hydrolysis. The monosaccharides were detected by high pressure anion exchange chromatography and pulsed amperometric detection. All three sugars were detected in each of the forms of mpl ligand. This result is indicative of the presence of sialic acid containing O-linked carbohydrate. This data correlates with the in vivo data as seen in FIG. 4 where CHO cell expressed forms of mpl ligand were all more active in vivo than the equivalent forms expressed in *E. coli*. Thus, the presence of carbohydrate enhances the in vivo activity of mpl ligand.

TABLE 3

### O-Linked Carbohydrate Calculations

Mpl Ligand Form	O-Glycanase Treatment (+/-)	Molecular Weight (Da)	Molecular Weight Shift	# of Potential O-Linked Chains (@950 Da/Chain)
1-332	-	54200	13600	14
	E. coli version	40600		
1-174	-	24600	8600	9
	+	16000		
1-163	-	18400	3900	4
	+	14500		
1-153	-	15200	2300	2
	+	12900		

20

### EXAMPLE 7

#### Mpl Ligand ELISA Assay

25 Polyclonal antibody production-- New Zealand White rabbits were hyperimmunized over a period of three

A-337B

- 36 -

months with recombinant human mpl ligand 1-163 produced in *E. coli*. Antisera from six rabbits exhibiting high antibody titers were pooled and specific anti-mpl ligand antibodies were affinity purified.

5           Affinity purification-- Recombinant human mpl ligand 1-163 was covalently attached to Actigel-ALD (Sterogene Bioseparations, Inc.) according to the manufacturer's instructions. An aliquot of the rabbit antisera pool was added to the mpl ligand affinity gel, 10 and the slurry was agitated gently on a rocker platform overnight a 4-8°C. Unbound serum proteins were washed from the gel bed with PBS and specifically bound anti-mpl ligand antibodies were then eluted with ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce Chemical Co.) 15 Recovered antibodies were dialyzed against several changes of PBS, then the antibody solution was concentrated in an Amicon stirred cell ultrafiltration unit and the resultant antibody concentrate was the source of specific anti-mpl ligand antibodies 20 subsequently used for well coating and enzyme conjugate preparations.

ELISA reagents--Immulon 4 Removawell Strips (Dynatech Laboratories, Inc.) were coated with affinity purified rabbit anti-mpl ligand antibodies. Affinity purified antibodies were diluted in 0.1 M sodium bicarbonate (freshly prepared pH about 8.2) to a concentration of 2.5 ug/ml. Each well received 100 ul of antibody and the plates were incubated for 24 hrs at room temperature in a sealed and humidified chamber. 25 Then, 200 ul of a blocking solution consisting of 1% fetal bovine serum 5% sucrose in TEN (50 mM Tris 7.4/10 mM EDTA/150 mM NaCl) was added to each well and plates were incubated and additional 24 hrs at room temperature in a sealed and humidified chamber. Combined coating 30 and blocking solutions were removed from the wells. An additional overcoating/blocking step was included: 300

A-337B

- 37 -

ul of SuperBlock Blocking Buffer in PBS (Pierce Chemical Co.) was added to each well. After standing at room temperature for about 5 min. this solution was removed and the wells were allowed to air dry at room

5 temperature for 24 hrs. The coated wells were stored in sealed plastic bags at 4-8°C until used in the mpl ligand ELISA.

Affinity purified anti-mpl ligand antibodies from a rabbit antisera pool were covalently coupled to

10 horseradish peroxidase (HRPO) for use as the signal generating antibody. The affinity purified antibodies were derivatized with iminothiolane HCl (Fluka Chemical Corp.). Separately, HRPO was derivatized with N-succinimidyl 6-maleimidocaproate (Fluka Chemical Corp.). The two activated proteins were combined to permit covalent coupling. The reaction mixture was then chromatographed down a FPLC Superose 6 (Pharmacia) column to isolate the antibody:HRPO conjugate of the desired molecular weight (i.e. about 200 kD). Fractions

15 containing the desired conjugate were combined and concentrated in a Centricon 30 (Amicon Division, W.R. Grace & Co.) and stored as a 50% glycerol solution at -20°C. This anti-mpl ligand Ab:HRPO concentrate was diluted into 2% fetal bovine serum in PBS for use in the

20 ELISA. The final concentration of conjugate used in the

25 ELISA was 250-500 ng/ml.

Recombinant human mpl ligand 1-163 produced in *E. coli* cells, was used for the preparation of standards. This mpl ligand was diluted into 2% fetal

30 bovine serum (Sigma Chemical Co.) in TEN buffer containing 0.05% thimerosal as a preservative. Standards prepared contained 1.0, 0.5, 0.25, 0.125 and 0.062 ng/ml mpl ligand.

Assay-100 ul of mpl ligand standards or

35 samples was added to wells then incubated for 18-24 hrs at room temperature in a sealed and humidified chamber.

A-337B

- 38 -

The well contents and residual solution were then removed and the wells washed once with wash solution (0.05% Tween 20 in TEN buffer). Anti-mpl ligand Ab:HRPO conjugate solution (100 ul) was added to each well and 5 then incubated for 2 hrs at room temperature in a sealed and humidified chamber. The contents of wells were removed then washed 4 times with 0.05% Tween 20 in TEN buffer.

For color development, 100 ul of TMB/peroxide 10 substrate solution (Kirkegaard & Perry Solutions A & B mixed 1:1) was added and incubated for 20 min at room temperature. The reaction was stopped by addition of 100 ul stop solution (0.5 N sulfuric acid) and the absorbance was read at 450 nm on microtiter plate 15 reader. Concentrations of mpl ligand in samples were calculated from a standard curve generated by using a curve fit program.

## EXAMPLE 8

20

Biological Activity of Mpl Ligand 1-174 Analogs in a Short-Term Liquid Culture Megakaryocyte Assay

Analogs of mpl ligand 1-174 were prepared as 25 described above and assayed for their ability to stimulate the growth of megakaryocytes in liquid culture. CD34 selected cells isolated from human leukapheresis units (Nichol et al., *Stem Cells* 12:494-505 (1994)) were plated at 2x10<sup>5</sup>/ml in culture 30 medium (IMDM / 1% Pen-Strep Glutamine / 1% Non-essential Amino Acids / 1% MEM Na-Pyruvate / 1% MEM Vitamins / 10% deionized BSA / 10% normal human AB plasma / 10 uM alpha-thiacylglycerol / 20. ug/ml L-Asparagine). In addition, 1.5 ul of COS-1 conditioned medium containing 35 mpl ligand (1-174) or mpl ligand 1-174 analog was added to each well. The final volume was 15 ul in Terasaki-

A-337B

- 39 -

style microtiter tissue culture plates (Vanguard International). Cells were incubated at 37°C for eight days in humidified boxes in 5% CO<sub>2</sub>, fixed directly to the culture wells with 1% glutaraldehyde, and then

5     incubated with a monoclonal antibody cocktail consisting of anti-GPIb, anti-GPIIb, (Biodesign) and anti-GPIb (Dako, Carpinteria, CA). The immune reaction was developed with a streptavidin-β-galactosidase detection system (HistoMark, Kirkegaard and Perry).

10    Megakaryocytes, identified by the darker color (blue in actual photographs), appear in FIG. 8.

Panels A and D of FIG. 8 are the positive and negative controls respectively. The well pictured in panel A received 37.5 pg of wild type mpl ligand 1-174

15    15    COS-1 conditioned medium and shows substantial megakaryocyte growth. Panel D received 1.5 ul of COS-1 mock conditioned medium and shows no growth. Panels B and C of FIG. 8 are mpl ligand 1-174 analogs N7 and N10 respectively. Panel B received 9.0 pg of mpl ligand

20    20    COS-1 conditioned medium while panel C received 27 pg and both show excellent megakaryocyte growth.

This experiment indicates that the analogs of mpl ligand tested are capable of stimulating the growth of human megakaryocytes in vitro.

25

#### EXAMPLE 9

##### Biological Activity of Mpl Ligand 1-174 Analogs in an In Vitro Cell Proliferation Assay

30

Analogs of mpl ligand 1-174 were prepared as described above and assayed for their ability to stimulate the proliferation of 32D-mpl cells. To construct 32D-mpl cells, the full length human mpl

35    35    receptor sequence (Vigon, I., et al., PNAS 89:5640-5644 (1992)) was subcloned into an expression vector

A-337B

- 40 -

containing the transcriptional promoter of Moloney Murine Sarcoma virus. Six ug of this construct and 6 ug of an amphotrophic retroviral packaging construct (Landau, N.R., Littman, D.R., *Journal of Virology* 56:5110-5113 (1992)) were transfected into  $3 \times 10^6$  293 cells using a CaPO<sub>4</sub> mammalian transfection kit (Stratagene). The same cells were retransfected after 2 days and again after 4 days. The day after the last transfection the 293 cells were cocultivated with the 10 IL-3 dependent murine cell line (32D, clone 23; Greenberger et al., *PNAS* 80:2931-2936 (1983)). After 24 hours, the 32D cells were rescued and banded in a BSA gradient (Path-o-cyte; Miles Inc.). Cells were expanded in 1 ng/ml murine IL-3 and then were selected for growth 15 in 20% APK9 serum (Bartley et al., *Cell* 77:1117-1124 (1994)). Cells were sorted for cell surface expression of receptor by FACS using a polyclonal rabbit antipeptide (MPL) serum. These cytokine dependent murine 32D-mpl cells are responsive to mpl ligand. 32D-MPL 20 cells were grown in MEM medium containing 10% Fetal Clone II Serum (Hyclone Laboratories) and 1.0 ng/ml muIL3 to a cell density of  $1 \times 10^6$  cells/ml. Cells were collected by centrifugation (approx. 500XG) and washed twice in growth medium lacking muIL3 and resuspended at 25  $1 \times 10^5$  cells/ml.

An extended twelve point mpl ligand standard curve was prepared using mpl ligand 1-163 and ranges from 5000 to 1 pg/ml. A volume of 100 ul of each dilution of standard mpl ligand or assay sample was 30 added to appropriate wells of a 96 well microtiter tissue culture plate containing 100 ul of resuspended cells (10,000 cells/well) and incubated in a humidified incubator at 37°C and 10% CO<sub>2</sub>. After 48 hours, 40 ul of MTS reagent (Aqueous Non-Radioactive Cell Proliferation 35 Kit, Promega) was added to each well and 14-18 hours later the plates were read on a plate reader at 490 nM.

A-337B

- 41 -

The *in vitro* activity in samples was calculated from a dose response curve for each sample. One unit was defined as the amount of mpl ligand in each sample required to give 50% of maximal stimulation. Specific 5 activity was calculated by dividing the biological activity in units/ml by the mpl ligand concentration in ng/ml as determined by mpl ligand ELISA.

The specific biological activity of mpl ligand analogs transfected and expressed in COS cells is shown 10 in Table 4. The effect of the amino acid substitutions on carbohydrate addition is also summarized. Purified human sequence mpl ligand has an *in vitro* activity that was 200-300 unit/ng as determined by the above-mentioned assays. It is apparent from Table 4 that mpl ligand 15 analogs containing additional N-linked carbohydrate are expressed as well as native sequence mpl ligand even when they contain additional carbohydrate chains (as determined in Example 6, Section A) e.g., N4 and N10. Both of these analogs retained full *in vitro* biological 20 activity also. Therefore the mpl ligand analogs containing N-linked carbohydrate can be expressed normally in mammalian cells and they can have normal or enhanced *in vitro* biological activity.

A-337B

- 42 -

TABLE 4

Mpl Ligand Form (Amino Acid Length)	Sequence	Number of N-linked chains	Elisa (ng/ml)	In Vitro Activity (units/ml)	Specific Activity (units/ng)
		(a)	(b)	(c)	(d)
MOCK	NONE	0	<0.08	<10	<125
N1 (174)	Native	NA	25	5375	215
N1 (174)	Native	0	31.4	8800	280
N1 (174)	Native	0	31.75	NA	NA
N2 (174)	N22	0	NA	NA	NA
N3 (174)	N25	NA	1.85	636	344
N4 (174)	N30T32	1	38	8830	232
N4 (174)	N30T32	1	24	NA	NA
N5 (174)	N38T40	0	1.2	<10	<8
N6 (174)	N86	0	0.44	<10	<22
N7 (174)	N82A83	0 to 1	6	2660	443
N7 (174)	N82A83	0 to 1	4.7	3080	655
N9 (174)	N92	0	10.5	1970	188
N10 (174)	N120T122	1	20.4	5943	291
N10 (174)	N120T122	1	33.7	9690	288
N11 (174)	S36N38T40	NA	<0.625	<10	<16
N11 (174)	S36N38T40	0	1.3	<10	<8
N13 (174)	N53T55	0 to 1	67	18000	269
N14 (174)	N58T60	0 to 1	17.9	4850	271
N15 (174)	N30T32N120T122	0 to 2	26	6420	247

5

## NOTES

(a) The number of additional N-linked chains was estimated based upon the mobility of the analog polypeptides in SDS gels as described in Example 6.

10

(b) Quantities of mpl ligand analogs in CHO cell supernatants were determined by ELISA assay as described in the Examples.

15

(c) In vitro activity was determined by measuring stimulation of thymidine uptake in 32D cells dependent on mpl ligand for growth.

A-337B

- 43 -

(d) Ratio of *in vitro* activity of mpl ligand analog as measured by proliferation assays to amount of mpl ligand analog measured by mpl ligand ELISA.

5

N.A. Not available.

#### EXAMPLE 10

10 Expression in CHO cells and Purification of Mpl Ligand 1-174, N4 and N15

15 pDSRA2 containing mpl ligand 1-174, N4 and N15 cDNA was transfected into DHFR-deficient CHO cells using the protocol described in Example 2 with the following modifications.

20 One transfection was performed for each analog. Three weeks after the transfection, mpl ligand expression was screened by mpl ligand ELISA. Three expressing clones for each form were frozen in cryogenic storage. The highest expressing clone for each analog was expanded for roller bottle production. For N4, 7.4 liters of conditioned medium (50% D-MEM, 50% HAMS-F12, 1% penicillin/ streptomycin/glutamine, 1% nonessential 25 amino acids (Gibco)) was produced and for N15, 4.6 liters of conditioned medium was produced.

30 Serum-free CHO cell conditioned medium from roller bottles seeded with CHO cells expressing mpl ligand 1-174 (2.9 L), N4 (7.4 L), N15 (4.4 L) was concentrated 12-, 19-, and 12- fold respectively using a S1Y10 (10,000 dalton molecular weight cut-off) Amicon Spiral ultrafiltration cartridge. One hundred fifty milliliters of concentrated conditioned medium was then loaded directly onto a 3.3 ml hu-MPL-X (receptor) 35 affinity column at a flow rate of 0.3 ml/min. The affinity column was constructed by coupling 1.0-1.5

A-337B

- 44 -

milligrams of Mpl-X (the soluble extra-cellular domain of the Mpl receptor) per milliliter of Pharmacia CNBr activated Sepharose resin as recommended by the manufacturer. After loading, the column was washed 5 with 30 ml of phosphate buffered saline (PBS; 10 mM NaPO<sub>4</sub> pH 6.8/150 mM NaCl) and then 60 ml of 10 mM Tris, pH 8.0 / 1M NaCl / 1 mM CHAPS. Mpl ligand 1-174 was eluted with 30 ml of 20 mM CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid) pH 10.5 / 1M NaCl / 1 mM CHAPS(3-10 [(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate).

Fractions were neutralized by adding 0.6 mL 1M Tris pH 7.0 to each eluted fraction. SDS-PAGE analysis showed an apparent "bleeding" of 1-174 mpl ligand during 15 the 10 mM Tris, pH 8.0 / 1M NaCl 1 mM CHAPS wash. Elution fractions were analyzed by SDS-PAGE. Those fractions containing mpl ligand 1-174 were pooled. This affinity purification was then modified and repeated with the following changes: 0.5 mL/min load and 20 elution, and the removal of the 10 mM Tris, pH 8.0 / 1M NaCl / 1 mM CHAPS wash.

All fractions containing the single mpl ligand band were concentrated using a YM10 (10,000 dalton molecular weight cut-off) membrane in a 50 mL stirred 25 cell, switching to a centricon device. This 0.5 mL concentrate was loaded directly onto a PBS equilibrated Pharmacia Superdex 200 HR 10/30 gel filtration column at 0.25 mL/min collecting 0.25 mL fractions. All eluted fractions containing a single mpl ligand band (based on 30 SDS-PAGE analysis) were pooled.

Other forms (N4 and N15) of CHO cell expressed mpl ligand were purified in a similar manner (two affinity purifications pooled and run on one Superdex 200 gel filtration column).

A-337B

- 45 -

## EXAMPLE 11

Determination of Carbohydrate Addition  
for CHO Cell Expressed N4 and N15

5

In order to determine whether N-linked carbohydrate was contained in the mpl ligand forms expressed in CHO cells, conditioned medium was analyzed by SDS-PAGE Western blot as described in Example 6 with 10 the following modifications.

CHO D- Conditioned medium from roller bottles was used. Samples were loaded into the Centricon-10 centrifugal concentrators (Amicon, Beverly, MA.) and were spun at 6000 RPM for one hour in a Beckman J2-HS 15 centrifuge using a fixed angle rotor (JA 20.1). A volume of concentrated sample containing approximately 100 ng of the mpl ligand analog was loaded on a SDS PAGE gel together with SDS sample buffer (described in Example 6.) *E. coli* expressed mpl ligand MK 1-174 containing no 20 carbohydrate was also loaded. FIG. 9 shows differences in mobility that correlate with the expected amount of carbohydrate. The fastest mobility species, Met-Lys (1-174) *E. coli* mpl ligand, was followed by mpl ligand 1-174 (CHO), N4 (CHO), and N15 (CHO) in succession. See 25 FIG. 9. The most likely explanation for size increases relative to unglycosylated mpl ligand is additional O-linked carbohydrate on mpl ligand 1-174 (CHO), additional O-linked carbohydrate and one additional N-linked oligosaccharide on N4 (CHO), and additional 30 O-linked carbohydrate and two additional N-linked oligosaccharides on N15 (CHO).

In order to establish that the increase in molecular weight was indeed due to the addition of N-linked carbohydrate chains, the samples were treated 35 with N-glycanase to remove any N-linked carbohydrate as described in Example 6. Each sample contained

A-337B

- 46 -

approximately 100 ng of mpl ligand analog purified from conditioned medium.

Following treatment with N-glycanase the mobility of N4 (CHO) and of N15 (CHO) was reduced to 5 that of mpl ligand 1-174 (CHO). Treatment of mpl ligand MK 1-174 (*E. coli*) or mpl ligand 1-174 (CHO) with N-glycanase did not affect mobility since neither form was expected to contain any N-linked carbohydrate. Comparison of N-glycanase treatment versus no treatment 10 shows that the size difference for N4 corresponds to the size of one N-linked carbohydrate chain and the size difference for N15 corresponds to the size of two carbohydrate chains. Thus addition of N-linked glycosylation sites for these two mpl ligand forms 15 resulted in additional N-linked carbohydrate when these species were expressed in CHO cells. See FIG. 10.

#### EXAMPLE 12

20 In vitro Biological Activity of Mpl Ligand Analogs made in CHO cells

Purified mpl ligand and analogs expressed and purified from in CHO cells or *E. coli* cells were 25 analyzed for in vitro biological activity using the factor dependent cell line 32D-MPL and assay described in Example 9 except activity was calculated from a curve using mpl ligand 1-332 produced in CHO cells as standard. The specific in vitro biological activities 30 of the various forms are shown in Table 5. It is apparent from this Table that the mpl ligand analogs containing additional carbohydrate, which are expressed in CHO cells have in vitro biological activity.

A-337B

- 47 -

TABLE 5

## IN VITRO ACTIVITY OF MPL LIGANDS

5

MPL LIGAND FORM	No. CHAINS	N-Linked IN VITRO ACTIVITY U/mg X10E6
MK174( E. coli)	0	13
1-163 (CHO)	0	86
1-174 (CHO)	0	85
N4 (CHO)	1	60
N15 (CHO)	2	92
1-332(CHO)	6	41

## EXAMPLE 13

10

In vivo Biological Activity of Mpl Ligand Analogs

Platelet counts from mice treated with various forms of mpl ligand were measured and the results are 15 presented in FIG. 11. CHO-derived mpl ligand 1-332, 1-174, N4, and N15 were produced and purified by mpl-receptor affinity chromatography. E. coli-derived Met-Lys-mpl ligand 1-174, was produced and purified by conventional chromatography. The indicated

A-337B

- 48 -

concentration of each form was administered subcutaneously into normal, female Balb/c mice once daily for 5 days. Test bleeds from a small lateral cut in a tail vein were collected 24 hours after the last 5 injection. Blood cell analyses were performed with a Sysmex electronic blood cell analyzer (Baxter Diagnostics, Inc. Irvine, CA). Data are represented as the mean of determinations of 4 animals, +/- standard error of the mean. Other blood cell parameters such as 10 total white blood cell counts or red blood cell counts were not affected by these treatments (data not shown).

All the forms stimulated increases in platelet counts. However the activities of the different forms varied. The relative *in vivo* activity was mpl ligand MK 15 1-174 (*E. coli*) < mpl ligand 1-174 (CHO) < N4 (CHO) < mpl ligand 1-332 (CHO) < N15 (CHO). The results indicate that addition of non-naturally occurring N-linked carbohydrate results in increased *in vivo* activity. It indicates further that increases in the 20 amount of carbohydrate result in proportional increases in *in vivo* activity.

## EXAMPLE 14

25 Construction of Mol Ligand Analogs and Truncations  
N16-N40 by Overlap PCR

Analogs N16 to N40 (see Table 6 for the 30 structures of these analogs) were constructed by overlap PCR (polymerase chain reaction) using a protocol adapted from Cheng et al., PNAS 91, 5695 (1994). Typically one to two mutations were introduced in each construction.

The following oligonucleotide primers were synthesized for use to prepare analogs N16-N40:

A-337B

- 49 -

5	5' F CCCTCTAGACCACCATGGAAC TGACTGAATTGCTCCTC	SEQ ID NO.: 18
	3' R (1-174) CCCGTCGACTCAGAGCTCGTTAGTGTG	SEQ ID NO.: 19
10	N16 - 3' R CCCGTCGACTCACTCCAACAATCCAGAAG	SEQ ID NO.: 20
	N17 - 3' R CCCGTCGACTTATCTGGCTGAGGCAGTGA	SEQ ID NO.: 21
	N18 - F CACGTCCTAACAGCAGCCTGAGCCAGTG	SEQ ID NO.: 22
	N18 - R CACTGGCTCAGGCTGCTGTTAAGGACGTG	SEQ ID NO.: 23
15	N19 - F CCCTTGCCCTAACGGTCCCTGCTGCCCTGCTGT	SEQ ID NO.: 24
	N19 - R ACAGCAGGCAGCAGGGAACCGTTAGGCAAAGGG	SEQ ID NO.: 25
	N20 - F TGCCTACACCTAACCTGTCGCCCTGCTGTGGA	SEQ ID NO.: 26
	N20 - R TCCACAGCAGGCAGCAGGGTAAAGGTGAGGCA	SEQ ID NO.: 27
20	N21 - F GGAAAACCAATATGTCGGAGACCAAGGCACA	SEQ ID NO.: 28
	N21 - R TGTGCCTTGGTCTCCGACATATTGGTTTCC	SEQ ID NO.: 29
25	N22 - F TGGGAGAACACACCACGATGGAGGAGACC	SEQ ID NO.: 30
	N22 - R GGTCTCCTCCATCGTGGTGTCCATTCTCCCA	SEQ ID NO.: 31
	N23 - F AAAACCCAGATGAACCGAGACGACCAAGGCACA	SEQ ID NO.: 32
	N23 - R TGTGCCTTGGTCTCGTTCATCTGGGTTT	SEQ ID NO.: 33
30	N24 - F CCCAGATGGAGAACACCTCGGCACAGGACAT	SEQ ID NO.: 34
	N24 - R ATGTCCCTGTGCCGAGGTGTTCTCCATCTGGG	SEQ ID NO.: 35
	N25 - F CACGGGGACAAAACGGAACCAACTGCCCTCTCA	SEQ ID NO.: 36
	N25 - R TGAGAGGCAAGTGGTCCGTTTGTCCCCGTG	SEQ ID NO.: 37
35	N26 - F CAGGGCAGGAACACATCTCACAGGATCCCA	SEQ ID NO.: 38
	N26 - R TGGGATCCTTGTGAGATGTGTTCTGCCCTG	SEQ ID NO.: 39
40	N27 - F GGGCAGGACCAACGCTAGCAAGGATCCCAAT	SEQ ID NO.: 40
	N27 - R ATTGGGATCCTTGTGCTAGCGTTGGTCTGCC	SEQ ID NO.: 41
	N29 - F pair1 CAGTGCAACGAGTCCCACCCCTGG	SEQ ID NO.: 42
	N29 - R pair1 CAAAGGGTGGGACTCGTTGCAGT	SEQ ID NO.: 43
45	N29 - F pair2 GACCACAAATCACTCGATCCCAA	SEQ ID NO.: 44
	N29 - R pair2 TTGGGATCGGAGTGATTGTGGTC	SEQ ID NO.: 45
	N30 - F GTCCCCACCAACACCTCTAGTCC	SEQ ID NO.: 46
	N30 - R GAGGACTAGAGAGGTGTTGGTGGGGAC	SEQ ID NO.: 47
50	N31 - 3' R CCCGTCGACTCACTCAGAAGCCCAGAGCCAGT	SEQ ID NO.: 48
	N36(1) - F GAAAACCCAGAACGAGACCAAGGCACAG	SEQ ID NO.: 49
	N36(1) - R CTGTGCCTTGGTGGTCTCGTTCTGGTTTC	SEQ ID NO.: 50

A-337B

- 50 -

	N36 (2) - F CACCAAGGCACAGGACATTCTGGGAG	SEQ ID NO.: 51
	N36 (2) - R CTCCCAGAATGTCCTGTGCCTGGTG	SEQ ID NO.: 52
5	N37 - F GAAAACCCAGATGAACGAGACCAAGGCACAG	SEQ ID NO.: 53
	N37 - R CTGTGCCTTGGTCTCGTCATCTGGGTTTC	SEQ ID NO.: 54
	N38 - F GTCCCCACCAACACCACTCTAGTCCTC	SEQ ID NO.: 55
10	N38 - R GAGGACTAGAGTGGTGTGGTGGGGAC	SEQ ID NO.: 56

F = Forward

R = Reverse

Constructions that introduce one new glycosylation site were performed in two successive steps. In step 1, two reactions were performed using 4 different oligonucleotides. These oligos included a 5' forward primer, a reverse mutagenic primer, a forward mutagenic primer (usually complementary to the reverse mutagenic primer) and a reverse 3' primer. The reverse 3' primer contained sequences that introduced stop codons followed by SalI restriction sites. Stop codons were introduced at positions 175, 184, 192, and 200. Thus, forms of lengths 1-174, 1-183 (N16), 1-191 (N17), and 1-199 (N31) could be made. PCR1 used template DNA (pDSR $\alpha$ 2 containing mpl ligand 1-174 sequences or full length mpl ligand 1-332 sequences), the 5' forward primer and the reverse mutagenic primer. PCR2 used template DNA, the 3' reverse primer and the forward mutagenic primer. The two PCR reactions were then performed and the amplified DNA fragments were separated by agarose gel electrophoresis. Small pieces of agarose containing DNA fragments of the correct size were excised from the gel.

35 The DNA fragments from PCR1 and PCR2 were combined together and a third PCR reaction was performed using only the 5' forward and 3' reverse primers. Thus, a full length DNA segment containing the desired mutations inserted into mpl ligand was amplified.

The amplified fragments were again separated by agarose gel electrophoresis, the correct sized DNA fragment was purified using a Geneclean™ kit and procedures supplied by the manufacturer (Bio 101, Inc.).

- 5 The purified DNA was digested with XbaI and SalI, then it was purified again using the Geneclean™ kit. The fragment was then ligated into XbaI and SalI cut pDSRα2. Ligated DNA was precipitated with 2 volumes of ethanol in 0.3M NaOAc pH 5.2 in the presence of carrier tRNA and
- 10 transformed into *E. coli*. Clones were tested by restriction analysis and agarose gel electrophoresis to identify those containing the correctly sized DNA inserts. Purified plasmid DNA was then prepared and the mpl ligand insert was sequenced to confirm the presence
- 15 of the desired mutations and to ensure that no additional amino acid changes were introduced.

In several cases, two or more mutations were combined simultaneously, i.e., see N29, N33, N34, N35, N39 and N40. This could be done by introducing a new

- 20 substitution into DNA already containing a change. For example, N33 was made by introducing the N23 changes into N15. In this case the procedure above was performed by using N23 mutagenic primers and the N15 template DNA.

In another strategy, two changes could be

- 25 introduced simultaneously into template DNA. The template DNA could contain natural sequences or could contain sequences encoding mpl ligand forms already containing changes. In these cases step 1 involved 3 PCR reactions and 6 oligos. The oligos included a 5' forward
- 30 primer, 2 pairs of forward and reverse mutagenic primers, and a reverse 3' primer. Each pair of primers was complementary to each other and contained sequences designed to introduce one new glycosylation site.

PCR1 included template DNA, the 5' forward

- 35 primer and the reverse mutagenic primer from pair 1.

PCR2 included template DNA, the forward mutagenic primer

A-337B

- 52 -

from pair 1 and the reverse mutagenic primer from pair 2 where pair 2 primers are 3' to pair 1 primers. PCR3 included template DNA, the forward mutagenic primer from pair 2 and the reverse 3' primer.

5 DNA fragments from each PCR reaction were separated by agarose gel electrophoresis and excised as before. The 3 DNA fragments were then combined together and amplified by PCR again using only the 5' forward and 3' reverse primers.

10 The DNA segment encoding the entire gene of interest with sequences containing two new glycosylation sites was then purified, cut with XbaI and SalI, and ligated into XbaI and SalI cut pDSR $\alpha$ 2 as before.

15 Multiple mutations could also be combined by performing the PCR reactions on templates already containing mutations. For example, N39 was made by introducing N36 and N38 changes into N15 template DNA. This was done using a different set of primers (N36(2)) than that used to make N36 (N36(1)). See primers set 20 forth above. Both sets of primers introduced the same mutation.

Longer mpl ligand forms could also be made. Thus, N40 was made in a similar manner to N39 except the 3' reverse primer in PCR 3, (step 1) and the PCR primer 25 in step 2 was the primer used to make N31. This primer introduces a stop codon at position 200 followed by a SalI restriction site. In addition, the template DNA used for PCR 3 contained sequences encoding full length mpl ligand (1-332).

30 The typical PCR reaction mix contained: 4 ul each of forward and reverse primers (5 pm/ul), 1 ul template (50 ng), 10 ul of 5X LP buffer (100 mM Tricine pH 8.7/25% glycerol/425 mM KOAc), 10 ul dNTP stock (1 mM each of dATP, dTTP, dCTP, dGTP), 0.8 ul rtTh polymerase 35 (Perkin Elmer; 2.5 U/ul), and 2 ul Vent polymerase (NEB; 0.01 U/ul after 1:100 fresh dilution in 1X LP buffer).

A-337B

- 53 -

H<sub>2</sub>O was added to bring the final volume to 50 ul. All the components were added together in the order shown and the PCR was started when the temperature during the first cycle was above the 60°C by adding 1 ul of 50 mM

5 MgOAc. Reaction conditions were: 2 cycles of 94°C, 10 sec/45°C, 1 min/ 68°C, 5 min followed by 25 cycles of 94°C, 10 sec/55°C, 1 min/ 68°C, 5 min.

These general procedures were used to construct the mpl ligand analogs and truncations N16 to 10 N40 shown in Table 6. The DNA sequence changes for each of the forms are shown.

A-337B

- 54 -

TABLE 6  
MPL LIGAND ANALOGS HAVING SITES  
FOR N-LINKED CARBOHYDRATE CHAINS

Species No.	Analogs/ Substitution	Amino Acid	Sequence Changes
N16	(1-183); Thr <sup>184</sup> →Gly <sup>332</sup> deleted	ACA→TGA (stop codon)	
N17	(1-191); Thr <sup>192</sup> →Gly <sup>332</sup> deleted	ACT→TAA (stop codon)	
N18	His <sup>23</sup> , Arg <sup>25</sup> →Asn <sup>23</sup> , Ser <sup>25</sup>	CAC, AGA→AAC, AGC	
N19	Thr <sup>37</sup> , Pro <sup>38</sup> , Val <sup>39</sup> → Asn <sup>37</sup> , Gly <sup>38</sup> , Ser <sup>39</sup>	ACA, CCT, GTC →AAC, GGT, TCC	
N20	Val <sup>39</sup> , Leu <sup>41</sup> →Asn <sup>39</sup> , Ser <sup>41</sup>	GTC, CTG→AAC, TCG	
N21	Gln <sup>54</sup> , Glu <sup>56</sup> →Asn <sup>54</sup> , Ser <sup>56</sup>	CAG, GAG→AAT, TCG	
N22	Lys <sup>52</sup> , Gln <sup>54</sup> →Asn <sup>52</sup> , Thr <sup>54</sup>	AAA, CAG→AAC, ACG	
N23	Glu <sup>57</sup> →Asn <sup>55'(i)</sup> , Thr <sup>57</sup>	GAG→AAC(i), ACG	
N24	Glu <sup>57</sup> , Lys <sup>59</sup> →Asn <sup>57</sup> , Ser <sup>59</sup>	GAG, AAG→AAC, TCG	
N25	Leu <sup>81</sup> , Pro <sup>83</sup> →Asn <sup>81</sup> , Thr <sup>83</sup>	CTG, CCC→AAC, ACC	
N26	Thr <sup>118</sup> , Ala <sup>120</sup> →Asn <sup>118</sup> , Ser <sup>120</sup>	ACC, GCT→AAC, TCT	
N27	Thr <sup>119</sup> , His <sup>121</sup> →Asn <sup>119</sup> , Ser <sup>121</sup>	ACA, CAC→AAC, AGC	
N29	Pro <sup>30</sup> , Val <sup>32</sup> , Ala <sup>120</sup> , Lys <sup>122</sup> → Asn <sup>30</sup> , Ser <sup>32</sup> , Asn <sup>120</sup> , Ser <sup>122</sup>	CCA, GTT, GCT, AAG→ AAC, TCC, AAT, TCC	
N30	Ser <sup>163</sup> , Arg <sup>164</sup> →Thr <sup>163</sup> , Asn <sup>164</sup>	AGC, AGA→ACC, AAC	

A-337B

- 55 -

N31	(1-199); Trp <sup>200</sup> →Gly <sup>332</sup> deleted	TGG→TGA (stop codon)
N33	Pro <sup>30</sup> , Val <sup>32</sup> , Glu <sup>57</sup> , Ala <sup>120</sup> , Lys <sup>122</sup>	CCA, GTT, GAG, GCT, AAG → → Asn <sup>30</sup> , Thr <sup>32</sup> , Asn <sup>55'(i)</sup> , Thr <sup>57</sup> AAC, TCC, AAC(i), ACG, Asn <sup>120</sup> , Thr <sup>122</sup> AAT, TCC
N34	Pro <sup>30</sup> , Val <sup>32</sup> , Glu <sup>57</sup> , Ser <sup>163</sup> , Arg <sup>164</sup>	CCA, GTT, GAG, AGC, AGA → → Asn <sup>30</sup> , Thr <sup>32</sup> , Asn <sup>55'(i)</sup> , Thr <sup>57</sup> , AAC, TCC, AAC(i), ACG, Thr <sup>163</sup> , Asn <sup>164</sup> ACC, AAC
N35	N4+N23+N30+N31 (1-199)	- -
N36	Met <sup>55</sup> , Glu <sup>57</sup> →Asn <sup>55</sup> , Thr <sup>57</sup>	ATG, GAG→AAC, ACC
N37	Glu <sup>56</sup> →Asn <sup>56</sup>	GAG→AAC
N38	Ser <sup>163</sup> , Arg <sup>164</sup> , Ser <sup>166</sup> →Thr <sup>163</sup> , Asn <sup>164</sup> , Thr <sup>166</sup>	AGC, AGA, TCT →ACC, AAC, ACT
N39	N4+N10+N36+N38 (1-174)	- -
N40	N4+N10+N36+N38+N31 (1-199)	- -

The symbol "(i)" in the above Table means that the referenced amino acid has been inserted. For example, Glu<sup>57</sup>→Asn<sup>55'(i)</sup>, Thr<sup>57</sup> (analog N23 in Table 6) means that the Glu at position 57 has been replaced with a Thr and, additionally, an Asn has been inserted just after the Met at position 55, and the Asn has been numbered 55' so that subsequent amino acids retain their previously assigned numbers.

Examples that include all changes from previous Examples are indicated by the specific analog

A-337B

- 56 -

numbers joined by "+" signs. See analogs N35, N39, and N40. The lengths of the amino acid chains of these analogs are indicated parenthetically. Thus, analog N35 contains a combination of all changes made for analogs 5 N4, N23, N30 and N31. The changes indicated for N31 mean that analog N35 is 199 amino acids long. All analogs in Table 6 are 174 amino acids long, except where indicated to be a different length (or, in the cases where an amino acid has been inserted, the total 10 length will be increased by the number of inserted amino acids).

## EXAMPLE 15

15 Characterization of Mpl Ligand Analogs and Truncations  
N16 to N40

A. Determination of expression level and in vitro biological activity of mpl ligand analogs.

20 Species N16 to N40 were transfected into COS cells using either the electroporation method (Example 5) or the CaPO4 method (Mammalian cell Transfection Kit; Specialty media). Cell free conditioned medium was 25 collected after 3-4 days, aliquoted and stored at -70 °C. Expression level was determined by ELISA assay as described in Example 7. The supernatants were also assayed for biological activity as described in Example 9 with one modification. The activities were calculated 30 from a standard curve using purified CHO cell expressed mpl ligand 1-332 as standard.

The results are shown in Table 7. As shown in Table 7 most of the mpl ligand analogs were expressed and secreted. Some of the analogs appeared to have 35 increased secretion. Bioassays on these samples indicated that the specific activities for most were

A-337B

- 57 -

also comparable to unmodified forms. Some of the analogs contained multiple N-linked carbohydrate chains (see below). This indicates that carbohydrate addition can result in increased secretion and normal *in vitro* 5 activity of the analogs.

TABLE 7

Mpl Ligand Form (Amino Acid Length)	Sequence	Number of N-linked chains	Elisa (ng/ml)	In Vitro Activity (units/ml)	Specific Activity (units/ ng)
		(a)	(b)	(c)	(d)
N1 (174)	Native	0	28	3991	143
N15 (174)	N30T32N120T122	0-2	45	7003	156
N16 (183)	1-183	0	85	9276	NA
N17 (191)	1-191	NA	<0.3	11	NA
N18 (174)	N23S25	0	2	5	2.5
N19 (174)	N37G38S39	NA	<0.3	NA	NA
N20 (174)	N39S41	NA	<0.3	<10	NA
N21 (174)	N54S56	0-1	30	4380	146
N22 (174)	N52T54	0-1	2	856	428
N23 (174)	N55'(I)T57	1	11	1058	96
N24 (174)	N57S59	0	5.3	458	86
N25 (174)	N81T83	NA	0.22	123	559
N26 (174)	N118S120	NA	0.9	96	106
N27 (174)	N119S121	0	4.5	338	75
N29 (174)	N30S32N120S122	0-2	15	1627	108
N30 (174)	T163N164	0-1	128	15592	122
N31 (199)	1-199	at least 1	156	19000	122
N33 (174)	4+10+23	3	78	10057	129
N34 (174)	4+23+30	at least 2	112	13536	120
N35 (198)	34 +31	4 or more	172	13112	76
N36 (174)	N55T57	0-1	48	5808	121
N37 (174)	N58	1	32	4504	141
N38 (174)	T163N164T166	0-1	25	3904	156
N39 (174)	N4+N10+N36+N38	3 to 4	127	17661	139
N40 (199)	N4+N10+N36+N38+N31	at least 5	134	19735	147

A-337B

- 58 -

## NOTES

5 (a) The number of additional N-linked chains was estimated according to the mobility of the analog polypeptides in SDS gels.

10 (b) Quantities of mpl ligand analogs in COS cell supernatants were determined by EIA.

15 (c) In vitro activity was determined by measuring proliferation of 32D-MPL cells which are dependent on mpl ligand for growth.

20 (d) Ratio of in vitro activity of mpl ligand analog as measured by proliferation assays to amount of mpl ligand analog measured by mpl ligand ELISA.

i- Insertion  
NA Not available

B. Determination of carbohydrate addition.

25 The analogs shown in Table 6 were tested to see if they added N-linked carbohydrate using the procedures described in Example 6.

30 Some analogs (N21, N22, N30, N33, and N36) were also tested with a modified procedure. This was necessary because the monoclonal antibody used to develop the Western blot was raised to a peptide including amino acid residues 47 to 62, and some of the analogs described in Table 6 contain substitutions that 35 affected immunoreactivity with this antibody, e.g. N21. Therefore, to analyze these analogs the supernatants were immunoprecipitated using a monoclonal antibody raised in mice to *E. coli* cell expressed mpl ligand

A-337B

- 59 -

1-163.

Typically 15 ugs of antibody was used to immunoprecipitate 50 ng of mpl ligand analog. Western blots with immunoprecipitated material were performed as 5 described in Example 6 except the immunoprecipitated bands were visualized by incubating the blots with the rabbit anti-mpl ligand polyclonal antibody (typically 1 ug/ml; raised to *E. coli* cell expressed mpl ligand 1-163) and an anti rabbit ECL kit (Amersham). The 10 results of the various experiments are shown in Table 7. Some of the analogs had increased size indicative of the presence of N-linked carbohydrate (N21, N22, N23, N29, N30, N31, N33, N34, N35, N36, N38, N39, and N40). A subset of these analogs had more than 1 N-linked chain, 15 e.g., N29, N33, N34, N35, N39 and N40. These analogs were secreted at normal or higher levels and had *in vitro* biological activity comparable to mpl ligand 1-174. This indicates that multiple functional N-linked glycosylation sites can be introduced into mpl ligand 20 without a deleterious effect on either expression or biological activity.

To demonstrate that multiple oligosaccharide chains can be added to mpl ligand, various analogs expressed in COS cells were analyzed by Western blot as 25 described in Example 6. FIG. 12 shows that the mobility of the analogs decreases with increasing numbers of added N-linked glycosylation sites. Analogs with 4 new sites are shown, N39 and N40. The analogs with the most N-linked sites had the slowest mobility. This result is 30 observed with both 1-174 and 1-199 forms of mpl ligand. This indicates that at least 4 analogs can be combined together resulting in new analogs with multiple N-linked carbohydrate chains.

A-337B

- 60 -

## EXAMPLE 16

Comparison of Glycosylation Sites Containing Asn-X-Ser  
vs. Asn-X-Thr

5

N-linked glycosylation sites include either Asn-X-Thr or Asn-X-Ser where X can be any one of the 20 naturally occurring amino acids except Pro. We wished to determine whether Ser or Thr is preferred in the third 10 position. Therefore, two sets of analogs with each set containing a mpl ligand glycosylation analog containing either a Ser or Thr at the third position in the sequon were examined to see if there was an effect on percent occupancy of the N-linked glycosylation sites. N15 15 contains 2 Asn-X-Thr sites while N29 contains 2 Asn-X-Ser sites at exactly the same positions. In a similar manner N30 contains an Asn-X-Ser while N38 contains an Asn-X-Thr at the same position.

To compare these two sets of analogs, they 20 were expressed in COS cells and the secreted mpl ligand was subjected to Western analysis as described in Example 6. FIG. 13 shows the results. N15 had a significantly increased proportion of glycosylated mpl ligand as compared to N29. In contrast, there was very 25 little difference in the proportion of glycosylated and unglycosylated mpl ligand when N30 and N38 were compared. These results indicate that both Asn-X-Ser and Asn-X-Thr can be introduced into mpl ligand and that both can act as sites for N-linked carbohydrate 30 addition. In addition, in some cases the Asn-X-Thr sequon may be preferred (i.e., it may be more efficiently glycosylated).

While the invention has been described in what 35 is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the

A-337B

- 61 -

contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such  
5 modifications and equivalents.

A-337B

- 62 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Elliott, Steven G.

(ii) TITLE OF INVENTION: MPL LIGAND ANALOGS

10

(iii) NUMBER OF SEQUENCES: 56

15

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: AMGEN INC.
- (B) STREET: 1840 Dehavilland Drive
- (C) CITY: Thousand Oaks
- (D) STATE: CA
- (E) COUNTRY: USA
- (F) ZIP: 91320-1789

20

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release 1.0, Version #1.30

25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

30

## (2) INFORMATION FOR SEQ ID NO:1:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1342 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 36..1094

50

(ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION: 1..98

55

(ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 99..1094

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

A-337B

- 63 -

	CAGGGAGCCA CGCCAGCCAA GACACCCCGG CCAGA ATG GAG CTG ACT GAA TTG Met Glu Leu Thr Glu Leu -21 -20	53
5	CTC CTC GTG GTC ATG CTT CTC CTA ACT GCA AGG CTA ACG CTG TCC AGC Leu Leu Val Val Met Leu Leu Leu Thr Ala Arg Leu Thr Leu Ser Ser 15 -10 -5 1	101
10	CCG GCT CCT CCT GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg 5 10 15	149
15	GAC TCC CAT GTC CTT CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val His 20 25 30	197
20	CCT TTG CCT ACA CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly 35 40 45	245
25	GAA TGG AAA ACC CAG ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly 50 55 60 65	293
30	GCA GTG ACC CTT CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG Ala Val Thr Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu 70 75 80	341
35	GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val 85 90 95	389
40	CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG CTT CCT Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu Pro 100 105 110	437
45	CCA CAG GGC AGG ACC ACA CCT CAC AAG GAT CCC AAT GCC ATC TTC CTG Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu 115 120 125	485
50	AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG CGT TTC CTG ATG CTT GTA Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu Val 130 135 140 145	533
55	GGA GGG TCC ACC CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA GCT GTC Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val 150 155 160	581
60	CCC AGC AGA ACC TCT CTA GTC CTC ACA CTG AAC GAG CTC CCA AAC AGG Pro Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn Arg 165 170 175	629
65	ACT TCT GGA TTG TTG GAG ACA AAC TTC ACT GCC TCA GCC AGA ACT ACT Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg Thr Thr 180 185 190	677

A-337B

- 64 -

	GGC TCT GGG CTT CTG AAG TGG CAG CAG GGA TTC AGA GCC AAG ATT CCT	725
	Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly Phe Arg Ala Lys Ile Pro	
	195 200 205	
5	GGT CTG CTG AAC CAA ACC TCC AGG TCC CTG GAC CAA ATC CCC GGA TAC	773
	Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly Tyr	
	210 215 220 225	
10	CTG AAC AGG ATA CAC GAA CTC TTG AAT GGA ACT CGT GGA CTC TTT CCT	821
	Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe Pro	
	230 235 240	
15	GGA CCC TCA CGC AGG ACC CTA GGA GCC CCG GAC ATT TCC TCA GGA ACA	869
	Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser Gly Thr	
	2 250 255	
20	TCA GAC ACA GGC TCC CTG CCA CCC AAC CTC CAG CCT GGA TAT TCT CCT	917
	Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr Ser Pro	
	260 265 270	
25	TCC CCA ACC CAT CCT CCT ACT GGA CAG TAT ACG CTC TTC CCT CTT CCA	965
	Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu Phe Pro Leu Pro	
	275 280 285	
30	CCC ACC TTG CCC ACC CCT GTG GTC CAG CTC CAC CCC CTG CTT CCT GAC	1013
	Pro Thr Leu Pro Thr Pro Val Val Gln Leu His Pro Leu Leu Pro Asp	
	290 295 300 305	
35	CCT TCT GCT CCA ACG CCC ACC CCT ACC AGC CCT CTT CTA AAC ACA TCC	1061
	Pro Ser Ala Pro Thr Pro Thr Ser Pro Leu Leu Asn Thr Ser	
	310 315 320	
40	TAC ACC CAC TCC CAG AAT CTG TCT CAG GAA GGG TAAGGTTCTC AGACACTGCC	1114
	Tyr Thr His Ser Gln Asn Leu Ser Gln Glu Gly	
	325 330	
45	GACATCAGCA TTGTCTCGTG TACAGCTCCC TTCCCTGCAG GGCGCCCTG GGAGACAACT	1174
	GGACAAGATT TCCTACTTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA TACACAGGAC	1234
	TGAAAAAGGGA ATCATTTTTC ACTGTACATT ATAAACCTTC AGAAGCTATT TTTTTAAGCT	1294
	ATCAGCAATA CTCATCAGAG CAGCTAGCTC TTTGGTCTAT TTCTGCA	1342
50	(2) INFORMATION FOR SEQ ID NO:2:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 353 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
55	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	

A-337B

- 65 -

Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu Leu Thr Ala  
 -21 -20 -15 -10

5 Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val  
 5 1 5 10

Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser  
 15 20 25

10 Gin Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala  
 30 35 40

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys  
 15 45 50 55

Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met  
 60 65 70 75

20 Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly  
 80 85 90

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu  
 95 100 105

25 Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp  
 110 115 120

30 Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val  
 125 130 135

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala  
 140 145 150 155

35 Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu  
 160 165 170

Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr  
 175 180 185

40 Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly  
 190 195 200

Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu  
 45 205 210 215

Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly  
 220 225 230 235

50 Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro  
 240 245 250

Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu  
 55 255 260 265

Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr  
 270 275 280

A-337B

- 66 -

10 Gly

(2) INFORMATION FOR SEQ ID NO:3:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 600 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

25 (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 12..596

30 (ix) FEATURE:  
(A) NAME/KEY: sig\_peptide  
(B) LOCATION: 12-74

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

40 TCTAGACCAC C ATG GAG CTG ACT GAA TTG CTC CTC GTG GTC ATG CTT CTC  
                   Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu  
                   -21 -20                   -15                   -10

CTA ACT GCA AGG CTA ACG CTG TCC AGC CCG GCT CCT CCT GCT TGT GAC  
Leu Thr Ala Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp  
15 5 1 5

CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAC GTC CTT CAC AGC  
 Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser  
 10 15 20

50 10 15 20  
 AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT GTC CTG 194  
 Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu  
 25 30 35 40

55 CTC CCT GCT GTG GAC TTT ACC TTG GGA GAA TGG AAA ACC CAG ATG GAG 242  
 Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu  
                   45                 50                 55

A-337B

- 67 -

	GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT CTG CTG GAG	290
	Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu	
	60 65 70	
5	GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC TCA TCC	338
	Gly Val Met Ala Ala Arg Gly Gin Leu Gly Pro Thr Cys Leu Ser Ser	
	75 80 85	
10	CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG GCC CTG	386
	Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu	
	90 95 100	
15	CAG AGC CTC CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC ACA GCT	434
	Gln Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala	
	105 110 115 120	
	CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG CTC CGA	482
	His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg	
	125 130 135	
20	GGA AAG GTG CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC CTC TGC GTC	530
	Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val	
	140 145 150	
25	AGG CGG GCC CCA CCC ACC ACA GCT GTC CCC AGC AGA ACC TCT CTA GTC	578
	Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val	
	155 160 165	
30	CTC ACA CTG AAC GAG CTC TAGG	600
	Leu Thr Leu Asn Glu Leu	
	170	

## (2) INFORMATION FOR SEQ ID NO:4:

35	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 195 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
40	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
45	Met Glu Leu Thr Glu Leu Leu Val Val Met Leu Leu Leu Thr Ala	
	-21 -20 -15 -10	
	Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val	
	5 1 5 10	
50	Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser	
	15 20 25	
	Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala	
55	30 35 40	
	Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys	
	45 50 55	

A-337B

- 68 -

Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met  
60 65 70 75

5 Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly  
80 85 90

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu  
95 100 105

10 Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp  
110 115 120

15 Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val  
125 130 135

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala  
140 145 150 155

20 Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu  
160 165 170

Asn Glu Leu

25 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
30 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
35 (A) DESCRIPTION: /desc = "nucleic acid"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
CCCCATGTCAA TCACAGCAGA CT

22

(2) INFORMATION FOR SEQ ID NO:6:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

55 \*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

A-337B

- 69 -

CTTCACAGCA ACCTGAGCCA GT

22

(2) INFORMATION FOR SEQ ID NO:7:

5

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

20 CAGTGCAACG AGACCCACCC TTTC

24

(2) INFORMATION FOR SEQ ID NO:8:

25

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCTACAAAT GTCACCGCTGC CTGCT

25

40

(2) INFORMATION FOR SEQ ID NO:9:

45

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

55

CCCACTTGTA ACTCATCCCT C

21

(2) INFORMATION FOR SEQ ID NO:10:

A-337B

- 70 -

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

20 CAACTGAACG CCACTTGTCT CTCA

24

(2) INFORMATION FOR SEQ ID NO:11:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

35 ACTTGTCTCA ACTCCACCCCT GGGGGA

26

(2) INFORMATION FOR SEQ ID NO:12:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

55 CTCCTGGGGA ACCTTTCTGG A

21

(2) INFORMATION FOR SEQ ID NO:13:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid

A-337B

- 71 -

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:  
GACCACAAAT CACACCGATC CCAAT 25

15 (2) INFORMATION FOR SEQ ID NO:14:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:  
30 ACCCTTTGTC TACAAATGTC ACGCTGCCTG CT 32

(2) INFORMATION FOR SEQ ID NO:15:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
45 TCTCTAAAC CTCACGGGGG AGCTT 25

(2) INFORMATION FOR SEQ ID NO:16:  
50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

A-337B

- 72 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:  
TGGAAAAATC AGACGGAGGA GAC 23

10 (2) INFORMATION FOR SEQ ID NO:17:  
10 (i) SEQUENCE CHARACTERISTICS:  
10 (A) LENGTH: 25 base pairs  
10 (B) TYPE: nucleic acid  
10 (C) STRANDEDNESS: single  
10 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid  
15 (A) DESCRIPTION: /desc = "nucleic acid"

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:  
20 TGGAGGAGAA CAAGACACAG GACAT 25

25 (2) INFORMATION FOR SEQ ID NO:18:  
25 (i) SEQUENCE CHARACTERISTICS:  
25 (A) LENGTH: 38 base pairs  
30 (B) TYPE: nucleic acid  
30 (C) STRANDEDNESS: single  
30 (D) TOPOLOGY: linear

35 (iii) MOLECULE TYPE: other nucleic acid  
35 (A) DESCRIPTION: /desc = "nucleic acid"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:  
40 CCCTCTAGAC CACCATGGAA CTGACTGAAT TGCTCCTC 38

45 (2) INFORMATION FOR SEQ ID NO:19:  
45 (i) SEQUENCE CHARACTERISTICS:  
45 (A) LENGTH: 28 base pairs  
45 (B) TYPE: nucleic acid  
45 (C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: other nucleic acid  
55 (A) DESCRIPTION: /desc = "nucleic acid"

55 (ix) FEATURE:  
55 (A) NAME/KEY: -  
55 (B) LOCATION: complement (1..28)

A-337B

- 73 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

5 GTGTGACTTG CTCGAGACTC AGCTGCC 28

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

## (ix) FEATURE:

20 (A) NAME/KEY: -  
(B) LOCATION: complement (1..29)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

25 GAAGACCTAA CAACCTCACT CAGCTGCC 29

## (2) INFORMATION FOR SEQ ID NO:21:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

40 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..29)

## 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTGACGGAG TCGGTCTATT CAGCTGCC 29

## (2) INFORMATION FOR SEQ ID NO:22:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

A-337B

- 74 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CACGTCCTTA ACAGCAGCCT GAGCCAGTG

29

10 (2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "nucleic acid"

20

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (1..29)

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTGCAGGAAT TGTGGTCGGA CTCGGTCAC

29

30 (2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "nucleic acid"

40

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCCTTTGCCT AACGGTTCCC TGCTGCCTGC TGT

33

(2) INFORMATION FOR SEQ ID NO:25:

50

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "nucleic acid"

A-337B

- 75 -

(ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..33)

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

10 GGGAAACGGA TTGCCAAGGG ACGACGGACG ACA 33

(2) INFORMATION FOR SEQ ID NO:26:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

30 TGCCTACACC TAACCTGTCG CCTGCTGTGG A 31

(2) INFORMATION FOR SEQ ID NO:27:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

40 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..31)

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

50 ACGGATGTGG ATTGGACAGC GGACGGACACC T 31

(2) INFORMATION FOR SEQ ID NO:28:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

A-337B

- 76 -

(A) DESCRIPTION: /desc = "nucleic acid"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGAAAACCAA TATGTCGGAG ACCAAGGCAC A

31

10 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "nucleic acid"

20

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (1..31)

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCTTTTGGTT ATACAGCCTC TGGTTCCGTG T

31

30 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

40 (A) DESCRIPTION: /desc = "nucleic acid"

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TGGGAGAAATG GAACACCACG ATGGAGGAGA CC

32

(2) INFORMATION FOR SEQ ID NO:31:

50

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "nucleic acid"

A-337B

- 77 -

5 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..32)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:  
10 ACCCTCTTAC CTTGTGGTGC TACCTCCTCT GG 32

15 (2) INFORMATION FOR SEQ ID NO:32:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:  
25 AAAACCCAGA TGAACGAGAC GACCAAGGCA CA 32

30 (2) INFORMATION FOR SEQ ID NO:33:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
35 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

45 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..32)

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:  
50 TTTGGGTCT ACTTGCTCTG CTGGTCCGT GT 32

55 (2) INFORMATION FOR SEQ ID NO:34:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

A-337B

- 78 -

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

10 CCCAGATGGA GAAACACCTCG GCACAGGACA T  
10 (2) INFORMATION FOR SEQ ID NO:35:

31

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid  
20 (A) DESCRIPTION: /desc = "nucleic acid"

25 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..31)

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:  
30 GGGTCTACCT CTTGTGGAGC CGTGTCTGT A  
30 (2) INFORMATION FOR SEQ ID NO:36:

31

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
40 (ii) MOLECULE TYPE: other nucleic acid  
40 (A) DESCRIPTION: /desc = "nucleic acid"

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:  
45 CACGGGGACA AACCGGAACC ACTTGCCCTCT CA

32

50 (2) INFORMATION FOR SEQ ID NO:37:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

A-337B

- 79 -

(A) DESCRIPTION: /desc = "nucleic acid"

5 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..32)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:  
10 GTGCCCTGT TTTGCCTTGG TGAACGGAGA GT

32

(2) INFORMATION FOR SEQ ID NO:38:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

30 CAGGGCAGGA ACACATCTCA CAAGGATCCC A

31

(2) INFORMATION FOR SEQ ID NO:39:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

45 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

50 GTCCCGTCCT TGTGTAGAGT GTTCCTAGGG T

31

(2) INFORMATION FOR SEQ ID NO:40:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

A-337B

- 80 -

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = 'nucleic acid'.

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

10 GGGCAGGACC AACGCTAGCA AGGATCCAA T

31

(2) INFORMATION FOR SEQ ID NO:41:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = 'nucleic acid'.

25 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..31)

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

33 CCCGTCCTGG TTGGCATCGT TCCTAGGGTT A

31

(2) INFORMATION FOR SEQ ID NO:42:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = 'nucleic acid'.

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

49 CAGTGCAACG AGTCCCCACCC TTGG

24

50 (2) INFORMATION FOR SEQ ID NO:43:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

A-337B

- 81 -

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

5 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..24)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTCACGTTGC TCAGGGTGGG AAAC

24

15 (2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

30 GACCACAAAT CACTCCGATC CCAA

24

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

45 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..24)

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTGGTGTTA GTGAGGCTAG GGTT

24

(2) INFORMATION FOR SEQ ID NO:46:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

A-337B

- 82 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

5

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTCCCCACCA ACACCTCTCT AGTCCTC

27

(2) INFORMATION FOR SEQ ID NO:47:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25 (ix) FEATURE:

(A) NAME/KEY: -  
(B) LOCATION: complement (1..27)

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CAGGGGTGGT TGTGGAGAGA TCAGGAG

27

(2) INFORMATION FOR SEQ ID NO:48:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

45

(ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..33)

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TGACCGAGAC CGGAAGACTT CACTCAGCTG CCC

33

55 (2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs

A-337B

- 83 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (iii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GAAAACCCAG AACGAGACCA CCAAGGCACA G

31

15 (2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (iii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

25

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (1..31)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CTTTTGGGTC TTGCTCTGGT GGTTCCGTGT C

31

35 (2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (iii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

45

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CACCAAGGCA CAGGACATTC TCGGAG

26

55 (2) INFORMATION FOR SEQ ID NO:52:

• (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid

A-337B

- 84 -

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

10 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

15 15 GTGGTTCCGT GTCCTGTAAG ACCCTC. 26

(2) INFORMATION FOR SEQ ID NO:53:

20 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 25 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

30 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:  
GAAAACCCAG ATGAAACGAGA CCAAGGCACA G 31

35 35 (2) INFORMATION FOR SEQ ID NO:54:

40 40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 45 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

50 50 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

55 55 CTTTTGGGTC TACTTGCTCT GGTTCCGTGT C 31

(2) INFORMATION FOR SEQ ID NO:55:

A-337B

- 85 -

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

15

15 GTCCCCACCA ACACCACTCT AGTCCTC

27

(2) INFORMATION FOR SEQ ID NO:56:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "nucleic acid"

30 (ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1..27)

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CAGGGGTGGT TGTGGTGAGA TCAGGAG

27

A-337B

- 86 -

WHAT IS CLAIMED IS:

1. An analog of mpl ligand comprising an amino acid sequence which includes at least one added, 5 at least one deleted or at least one altered glycosylation site.

2. The analog of Claim 1 wherein the glycosylation site is for an N-linked carbohydrate 10 chain.

3. The analog of Claim 1 wherein the glycosylation site is for an O-linked carbohydrate chain.

15 4. The analog of Claim 1 having at least one additional carbohydrate chain attached thereto as expressed in a eukaryote.

20 5. The analog of Claim 4 wherein the carbohydrate chain is an N-linked carbohydrate chain.

6. The analog of Claim 4 wherein the carbohydrate chain is an O-linked carbohydrate chain.

25 7. The analog of Claim 1 which is the product of expression of an exogenous DNA sequence in a eukaryote.

30 8. The analog of Claim 5 which is selected from the group consisting of

[Asn<sup>30</sup>, Thr<sup>32</sup>] mpl ligand;  
[Asn<sup>82</sup>, Ala<sup>83</sup>] mpl ligand;  
35 [Asn<sup>87</sup>, Thr<sup>89</sup>] mpl ligand;  
[Asn<sup>53</sup>, Thr<sup>55</sup>] mpl ligand;

A-337B

- 87 -

[Asn<sup>58</sup>, Thr<sup>60</sup>] mpl ligand;  
[Asn<sup>30</sup>, Thr<sup>32</sup>, Asn<sup>120</sup>, Thr<sup>122</sup>] mpl ligand;  
[Asn<sup>37</sup>, Gly<sup>38</sup>, Ser<sup>39</sup>] mpl ligand;  
[Asn<sup>39</sup>, Ser<sup>41</sup>] mpl ligand;  
5 [Asn<sup>54</sup>, Ser<sup>56</sup>] mpl ligand;  
[Asn<sup>52</sup>, Thr<sup>54</sup>] mpl ligand;  
[Asn<sup>55</sup>(i), Thr<sup>57</sup>] mpl ligand;  
[Asn<sup>81</sup>, Thr<sup>83</sup>] mpl ligand;  
[Asn<sup>118</sup>, Ser<sup>120</sup>] mpl ligand;  
10 [Asn<sup>30</sup>, Ser<sup>32</sup>, Asn<sup>120</sup>, Ser<sup>122</sup>] mpl ligand;  
[Thr<sup>163</sup>, Asn<sup>164</sup>] mpl ligand;  
[Asn<sup>30</sup>, Thr<sup>32</sup>, Asn<sup>120</sup>, Thr<sup>122</sup>, Asn<sup>55</sup>(i), Thr<sup>57</sup>] mpl ligand;  
[Asn<sup>30</sup>, Thr<sup>32</sup>, Asn<sup>55</sup>(i), Thr<sup>57</sup>, Thr<sup>163</sup>, Asn<sup>164</sup>] mpl ligand;  
[Asn<sup>55</sup>, Thr<sup>57</sup>] mpl ligand;  
15 [Asn<sup>56</sup>] mpl ligand;  
[Thr<sup>163</sup>, Asn<sup>164</sup>, Thr<sup>166</sup>] mpl ligand; and  
[Asn<sup>30</sup>, Thr<sup>32</sup>, Asn<sup>120</sup>, Thr<sup>122</sup>, Asn<sup>55</sup>, Thr<sup>57</sup>, Thr<sup>163</sup>,  
Asn<sup>164</sup>, Thr<sup>166</sup>] mpl ligand.

20 9. An analog of Claim 1, wherein the mpl ligand has an amino acid sequence selected from the group consisting of:

	mpl ligand 1-332	amino acids 1-332 of FIG. 1
	mpl ligand 1-199	amino acids 1-199 of FIG. 1
25	mpl ligand 1-191	amino acids 1-191 of FIG. 1
	mpl ligand 1-183	amino acids 1-183 of FIG. 1
	mpl ligand 1-174	amino acids 1-174 of FIG. 1
	mpl ligand 1-163	amino acids 1-163 of FIG. 1
	mpl ligand 1-153	amino acids 1-153 of FIG. 1
30	mpl ligand 1-152	amino acids 1-152 of FIG. 1
	mpl ligand 1-151	amino acids 1-151 of FIG. 1
	mpl ligand 7-332	amino acids 7-332 of FIG. 1
	mpl ligand 7-191	amino acids 7-191 of FIG. 1
	mpl ligand 7-199	amino acids 7-199 of FIG. 1
35	mpl ligand 7-183	amino acids 7-183 of FIG. 1
	mpl ligand 7-174	amino acids 7-174 of FIG. 1

A-337B

- 88 -

mpl ligand 7-163      amino acids 7-163 of FIG. 1  
mpl ligand 7-153      amino acids 7-153 of FIG. 1  
mpl ligand 7-152      amino acids 7-152 of FIG. 1  
mpl ligand 7-151      amino acids 7-151 of FIG. 1

5

10. An analog of human mpl ligand selected from the group consisting of:

[Asn<sup>22</sup>] mpl ligand;  
10      [Asn<sup>25</sup>] mpl ligand;  
[Asn<sup>38</sup>, Thr<sup>40</sup>] mpl ligand;  
[Asn<sup>86</sup>] mpl ligand;  
[Asn<sup>92</sup>] mpl ligand;  
[Asn<sup>120</sup>, Thr<sup>122</sup>] mpl ligand;  
15      [Ser<sup>36</sup>, Asn<sup>38</sup>, Thr<sup>40</sup>] mpl ligand;  
[Asn<sup>88</sup>, Thr<sup>90</sup>] mpl ligand;  
[Asn<sup>23</sup>, Ser<sup>25</sup>] mpl ligand;  
[Asn<sup>57</sup>, Ser<sup>59</sup>] mpl ligand; and  
[Asn<sup>119</sup>, Ser<sup>121</sup>] mpl ligand.

20

11. An analog of human mpl ligand according to Claim 10, wherein said mpl ligand is selected from the group consisting of:

25      mpl ligand 1-332      amino acids 1-332 of FIG. 1  
mpl ligand 1-199      amino acids 1-199 of FIG. 1  
mpl ligand 1-191      amino acids 1-191 of FIG. 1  
mpl ligand 1-183      amino acids 1-183 of FIG. 1  
mpl ligand 1-174      amino acids 1-174 of FIG. 1  
30      mpl ligand 1-163      amino acids 1-163 of FIG. 1  
mpl ligand 1-153      amino acids 1-153 of FIG. 1  
mpl ligand 1-152      amino acids 1-152 of FIG. 1  
mpl ligand 1-151      amino acids 1-151 of FIG. 1  
mpl ligand 7-332      amino acids 7-332 of FIG. 1  
35      mpl ligand 7-199      amino acids 7-199 of FIG. 1  
mpl ligand 7-191      amino acids 7-191 of FIG. 1

A-337B

- 89 -

	mpl ligand 7-183	amino acids 7-183 of FIG. 1
	mpl ligand 7-174	amino acids 7-174 of FIG. 1
	mpl ligand 7-163	amino acids 7-163 of FIG. 1
	mpl ligand 7-153	amino acids 7-153 of FIG. 1
5	mpl ligand 7-152	amino acids 7-152 of FIG. 1
	mpl ligand 7-151	amino acids 7-151 of FIG. 1

12. An analog according to any of Claims  
8-11, which is the product of expression of an exogenous  
10 DNA sequence in a eukaryotic cell.

13. An analog according to Claim 12, wherein  
said eukaryotic cell is a mammalian cell.

15 14. An analog of Claim 1, which is  
[Asn<sup>30</sup>, Thr<sup>32</sup>, Asn<sup>120</sup>, Thr<sup>122</sup>, Asn<sup>55'(i)</sup>, Thr<sup>57</sup>, Thr<sup>163</sup>,  
Asn<sup>164</sup>, Thr<sup>166</sup>] mpl ligand 1-174; or  
[Asn<sup>30</sup>, Thr<sup>32</sup>, Asn<sup>120</sup>, Thr<sup>122</sup>, Asn<sup>55'(i)</sup>, Thr<sup>57</sup>, Thr<sup>163</sup>,  
Asn<sup>164</sup>, Thr<sup>166</sup>] mpl ligand 1-199,  
20 which is the product of expression of an exogenous DNA  
sequence in a eukaryotic cell.

15. An analog according to Claim 14, wherein  
the eukaryotic cell is mammalian.

25 16. An analog according to Claim 15, wherein  
said mammalian cell is CHO.

17. An analog of mpl ligand comprising an  
30 amino acid sequence which comprises (a) substitution of  
any of the O-linked carbohydrate sites in mpl ligand  
with an N-linked carbohydrate site; (b) substitution of  
any of the N-linked carbohydrate sites in mpl ligand  
with an O-linked carbohydrate site; (c) substitution of  
35 any of the O-linked carbohydrate sites in mpl ligand  
with a different O-linked carbohydrate site; or (d)

A-337B

- 90 -

substitution of any of the N-linked carbohydrate sites in mpl ligand with a different N-linked carbohydrate site.

5 18. A DNA sequence encoding an analog of mpl ligand which includes at least one added, at least one deleted or at least one altered glycosylation site.

10 19. A DNA sequence encoding an analog of mpl ligand according to any of Claims 1-11 and 13-17.

20. A eukaryotic host cell transfected with a DNA sequence according to Claim 19 in a manner allowing the host cell to express an analog of mpl ligand.

15 21. A composition comprising a therapeutically effective amount of an mpl ligand analog according to any of Claims 1-11 and 13-17 together with a pharmaceutically acceptable diluent, adjuvant or  
20 carrier.

1	CAGGGAGGCCACGCCAAGACACCCCCGGCCAGAATGGAGGCTGACTGAAATTGCTCCCTC	53
-21	Met Glu Leu Thr Gln Leu Leu Leu	-14
70	GTGGCTCATGGCTTCTCCTAACTGCAAGGCTAACGGCTAACGGCTGTCAGCCCCGGCTCCTGCTTGT	119
-13	Val Val Met Leu Leu Thr Ala Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys	7
120	GACCTCCGGAGGTCCCTCAGTAACCTGCTTGGTGAACGCCATGCCATGCCATGCCACTGAGC	179
8	Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser	27
180	CAGTCCCCAGAGGGTCACCCCTAACCTTGCTAACCTTGCTGCCCTGCTGTTGACTTGTAGC	239
28	Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Pro Ala Val Asp Phe Ser	47
240	TTGGAGAATGGAAACCCAGATGGAGGACCAAGGCACAGGGACATTCTGGAGGAGTG	299
48	Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Lys Ala Arg Gln Asp Ile Leu Gly Ala Val	67
300	ACCCCTTCTGGAGGGAGTGATGGCAGGCACGGGACAACTGGGACCCACTTGCCTCTCA	359
68	Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser	87
360	TCCCTCCCTGGGGCAGCTTCTGGACAGGTCCTCGTCTGGGGCCCTGCGAGGCCCTC	419
88	Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu	107

**FIG. 1A**

420	CTTGGAACCCAGCTTCCCTCACAGGGCAGGACCAAGGATCACAAGGATCCAAATGCCATCC	479
108	LeuGlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAsnProAsnAlaIle	127
480	TTCCTGAGGCTTCCAAACACCTGCTCCGAGGAAAGGTGGCTTCTGATGCTGTAGGAGGG	539
128	PheLeuSerPheGlnHisLeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGly	147
540	TCCACCCCTCTGGCTCAGGGGGCCCCACCCACACAGCTGTCCTCAGCAGAACCTCTCA	599
148	SerThrLeuCysValArgArgAlaProProThrThrAlaValProSerArgThrSerLeu	167
600	GTCCTCACACTGAAACGGACTTCTGGATTCTGGAGACAAACTTCACT	659
168	ValLeuThrLeuAsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThr	187
660	GCCTCAGCCAGAACTACTGGCTCTGGCTCTGAAAGTGGCAGCAGGGATTCAAGGCCAG	719
188	AlaSerAlaArgThrThrGlySerGlyLeuLeuLysThrGlnGlnGlyPheArgAlaLys	207
720	ATTCCCTGCTGCTGAACCAACCTCCAGGTCCCTGGACCAAATCCCCGGATAACCTGAAAC	779
208	IleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsn	227
780	AGGATAACGAAACTCTTGAATGGAAACTCGGACTCTCTGGACCTTCACGGAGGAC	839
228	ArgIleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr	247

840	CTAGGAGCCGGACATTCCCTCAGGAACATCAGAACACAGGCTCCCTGCCACCCAAACCTC	899
248	LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsnLeu	267
900	CAGGCCTGGATATTCTCCCTCCACCCATCCTCCCTACTGGACAGTATAAGCTCTTCCTC	959
268	GlnProGlyTyrrSerProSerProThrHisProProThrHisProProAspProSer	287
960	CTTCCACCCACCTTGCCACCCCTGTCGGTCCAGCTCCACCCCTGCTTGACCCCTTCT	1019
288	LeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuProAspProSer	307
1020	GCTCCAACGCCACCCCTTACCGCCCTCTCTAAACACATCCTACACCCACTCCCAGAAT	1079
308	AlaProThrProThrSerProLeuAsnThrSerThrSerGlnAsn	327
1080	CTGTCCTCAGGAAGGTAAGGTTCTCAGAACACTGCCGACATCAGCATTCGTCTCGTGTACAG	1139
329	LeuSerGlnGluGlyEnd	
1140	CTCCCTTCCCTGCAGGGGGCCCTGGAGACAACGGACAAAGATTCCCTACTTTCTCCCTG	1199
1200	AAACCCAAAGCCCTGGTAAAGGGATAACACAGGACTGAAAAGGGAAATCAATTTCACGT	1259
1260	ACATTATAAACCTCAGAAGCTATTAAAGCTATCAGCAATACTCATCAGGAGCCT	1319
1320	AGCTCTTGGTCTATTTCAGCA	1342

10	30	50		
· TCTAGACCACCATGGAGCTGACTGAAATTGCTCCTCGTGGTCATGCTTCTCTAACTGCAA				
M E L T E L L V V M L L T A R				
70	90	110		
· GGCTAACGCTGTCCAGCCCCGGCTCCTGCTTGTGACCTCCGAGTCCTCAGTAAACTGCC				
L T L S S P A P P A C D L R V L S K L L				
130	150	170		
· TTCTGTGACTCCACGTCCTCACAGCAGACTGGAGCCAGTGCCAGAGGTTCACCCCTTGC				
R D S H V L H S R L S Q C P E V H P L P				
190	210	230		
· CTACACCTGTCCTGGCTGGACTTTAGCTTGGAGAATGGAAAACCCAGATGG				
T P V L L P A V D F S L G E W K T Q M E				
250	270	290		
· AGGAGACCAAGGCACAGGACATTCTGGGAGGACTGACCCCTTCTGGCTGGAGGTGATGG				
E T K A O D I L G A V T L L E G V M A				

FIG. 2A

310 . . . . .  
 CAGCACGGGACAACTGGGACCCACTTGCCTCTCATCCCTCCTGGCAGCTTCTGGAC  
 A R G Q L G P T C L S S L L G Q L S G Q  
  
 370 . . . . .  
 AGGTCCGTCCTCCCTGGGCCCTGCAGAGCCTCCTTGGAAACCCAGCTTCCACAGG  
 V R L L G A L Q S L L G T Q L P P Q G  
  
 430 . . . . .  
 GCAGGACCAAGGCTCACAGGATCCCAATGCCATCTTCCATGAGCTTCCAACACCTGCTCC  
 R T T A H K D P N A I F L S F Q H L L R  
  
 490 . . . . .  
 GAGGAAAGGTGGTTCCTGATGGCTTGTAGGGGGTCCACCCCTCTGGCTCAGGGGGCC  
 G K V R F L M L V G G S T L C V R R A P  
  
 550 . . . . .  
 CACCCACAGGCTGTCCCCAGCAGAACCTCTAGTCCTCACACTGAACGAGCTCTAGG  
 P T T A V P S R T S L V L T L N E L \*  
 TCGAC

**FIG. 2B**

6/14

1	2	3	4	5	6	7	8	9	10	11	12
—	—	—	—	—	—	—	—	—	—	—	—

Mw  
(kDa) 95.5 —

55 —

43 —

36 —

29 —

18.4 —

12.4 —

Neuraminidase  
+ O-Glycanase  
Treatment

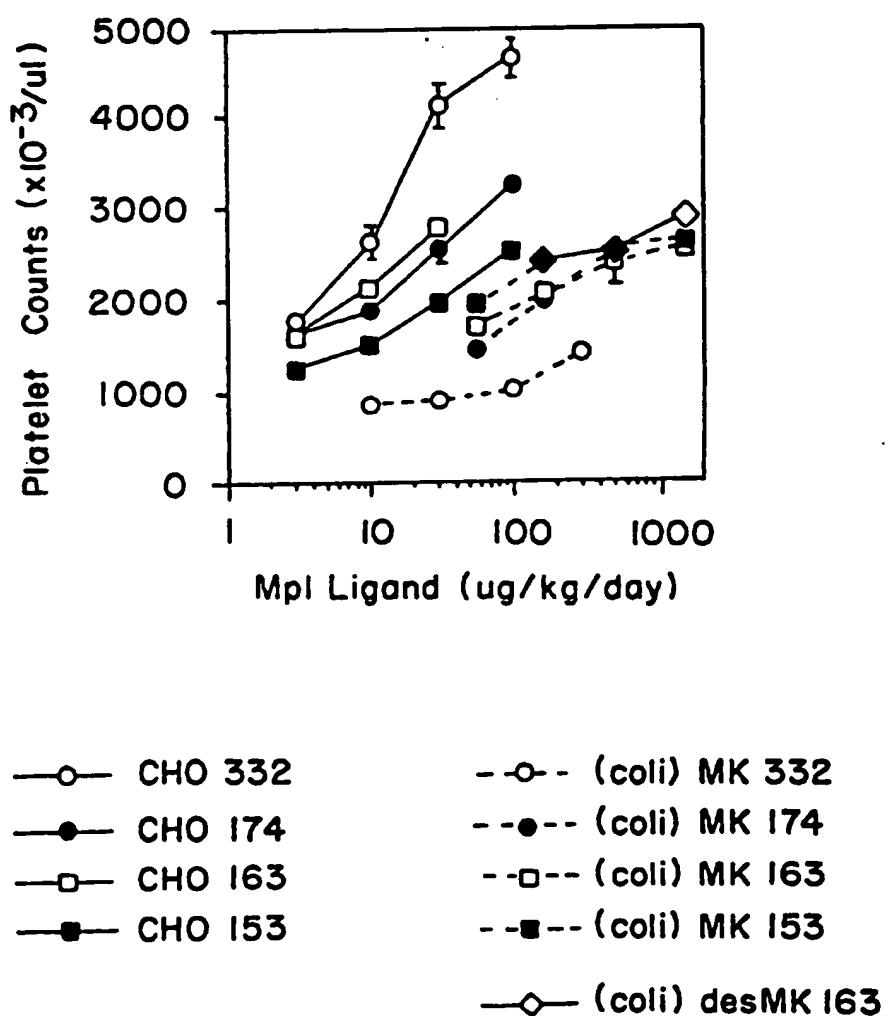
N-Glycanase  
Treatment

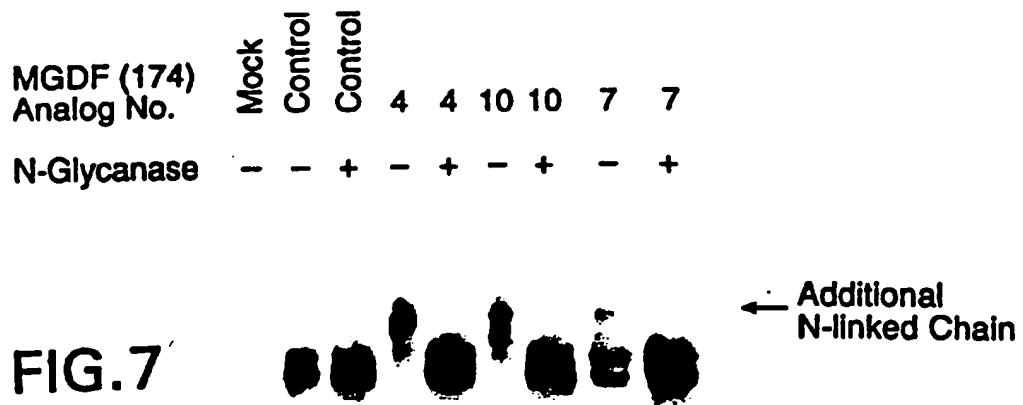
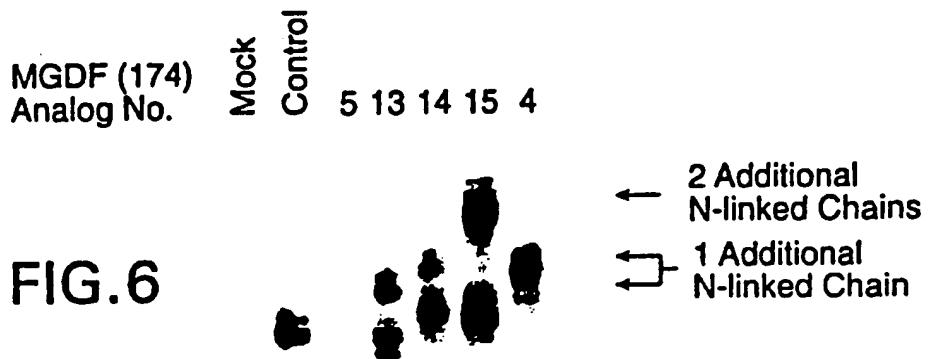
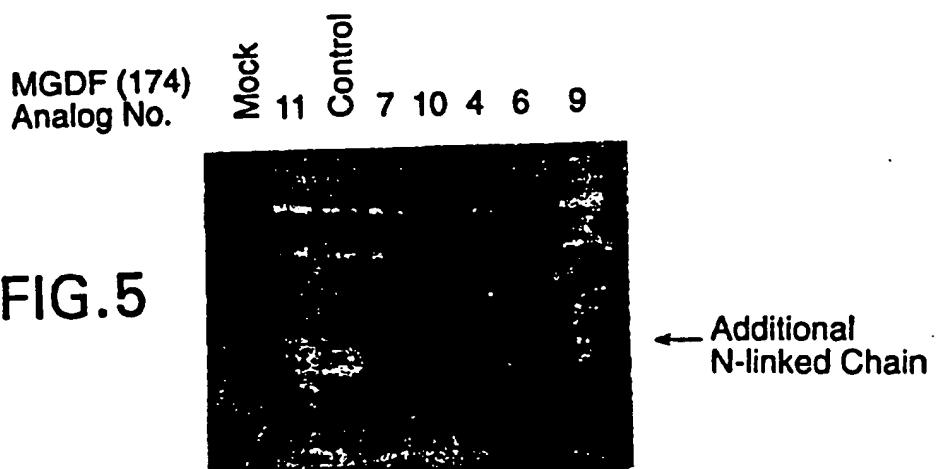
- 1. MGDF 1-332 (CHO)
- 2. MGDF 1-332 (CHO)
- 3. MGDF 1-332 (E. coli; MK)
- 4. MGDF 1-174 (CHO)
- 5. MGDF 1-174 (CHO)
- 6. MGDF 1-174 (E. coli; MK)
- 7. MGDF 1-163 (CHO)
- 8. MGDF 1-163 (CHO)
- 9. MGDF 1-163 (E. coli; desMK)
- 10. MGDF 1-153 (CHO)
- 11. MGDF 1-153 (CHO)
- 12. MGDF 1-153 (E. coli; MK)

FIG. 3

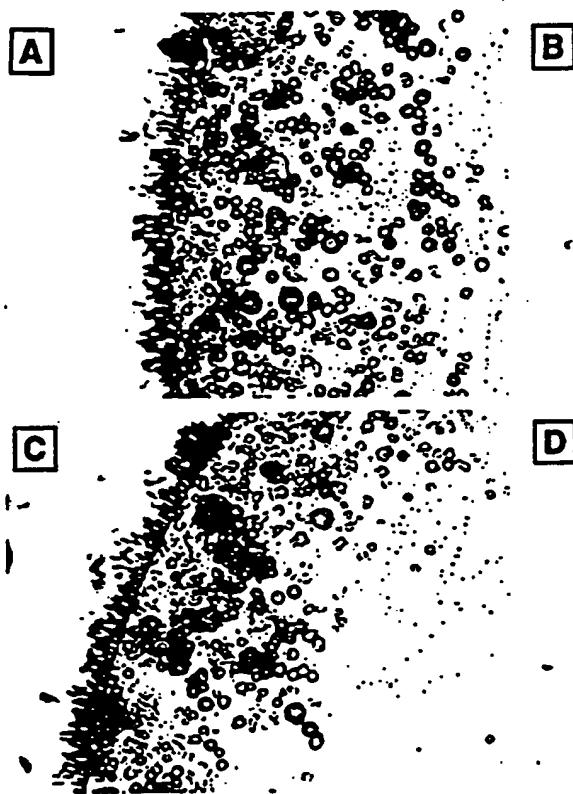
SUBSTITUTE SHEET (RULE 26)

FIG. 4

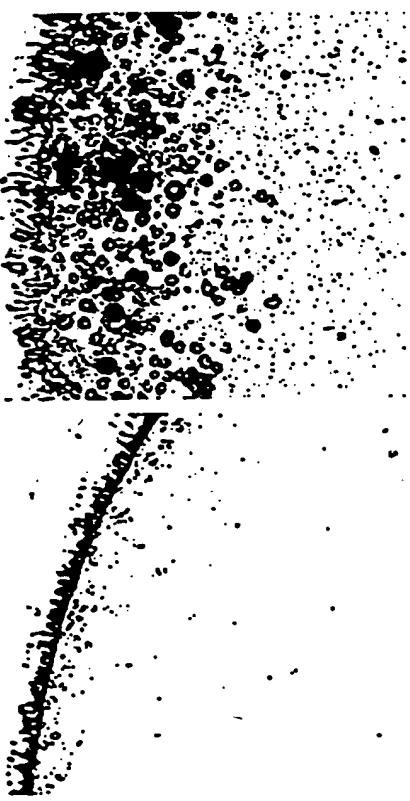




**FIG.8A**



**FIG.8B**



**FIG.8C**

**FIG.8D**

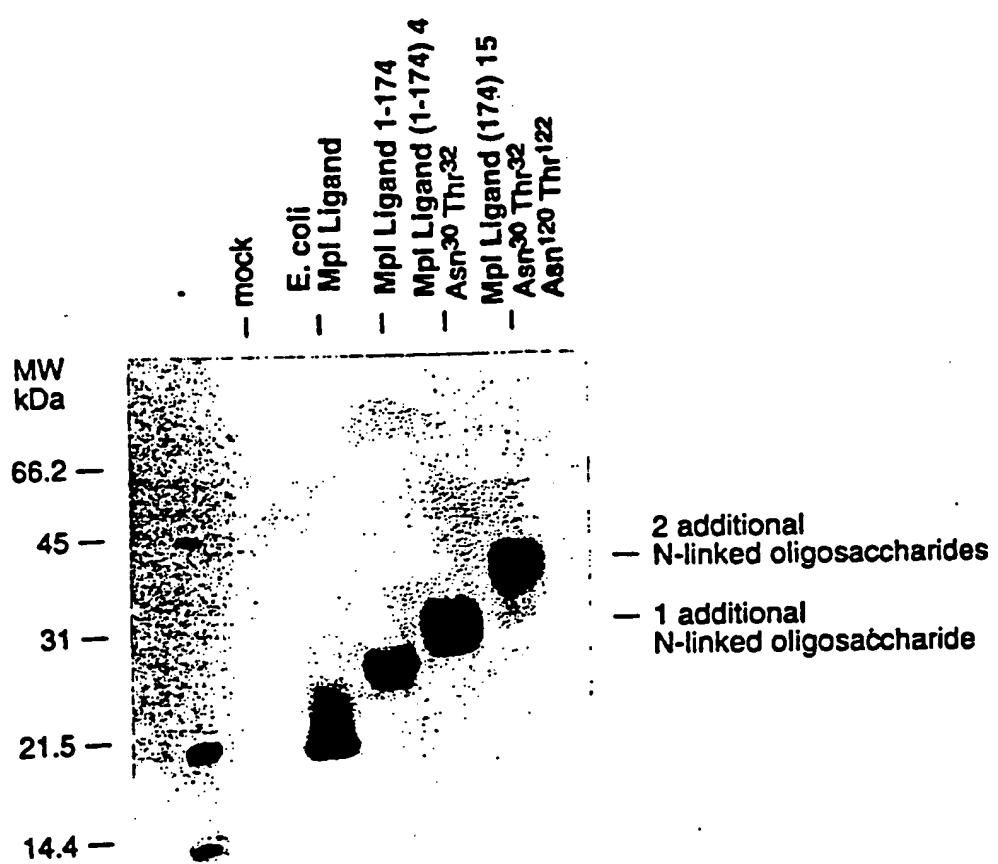


FIG. 9

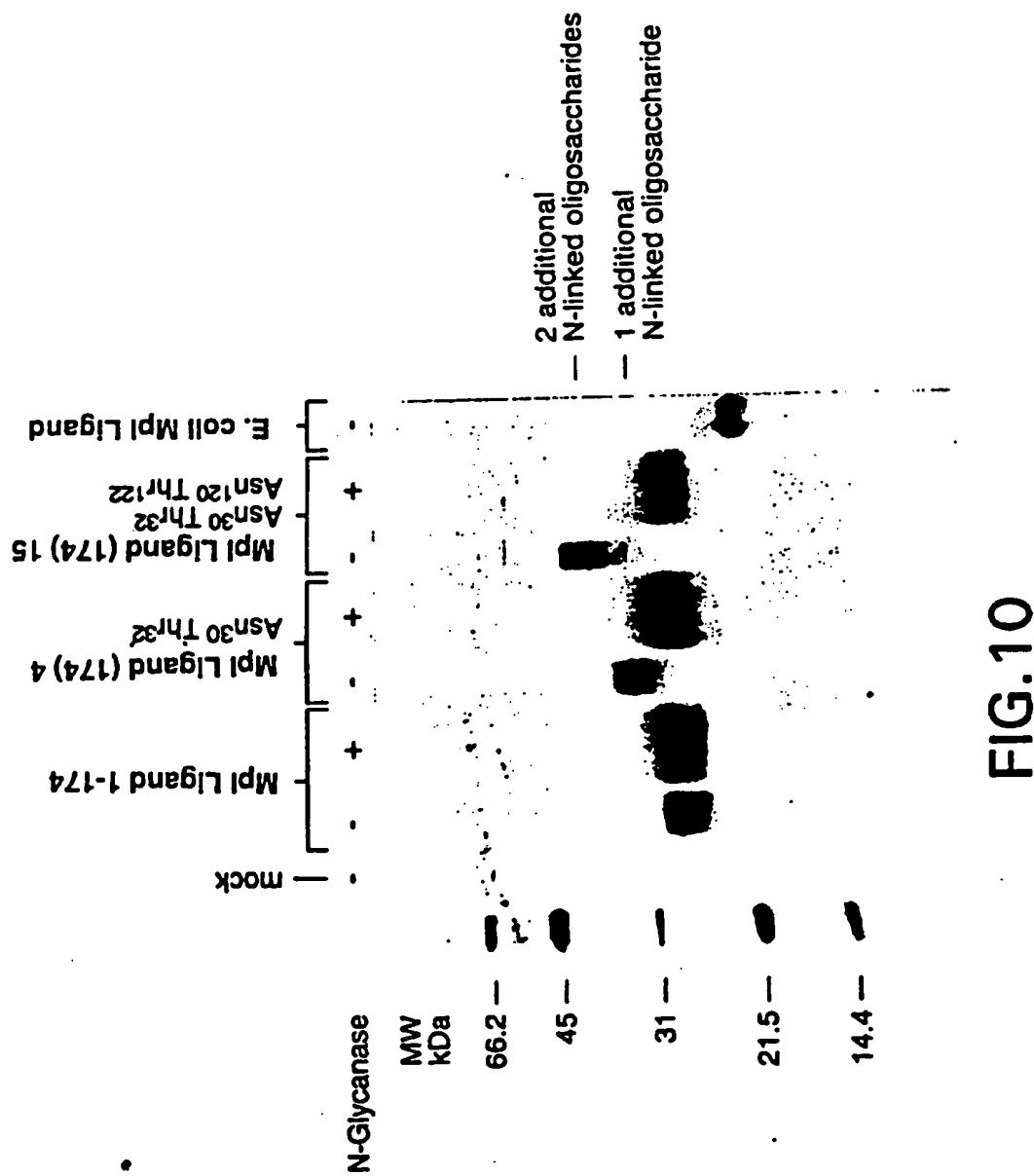
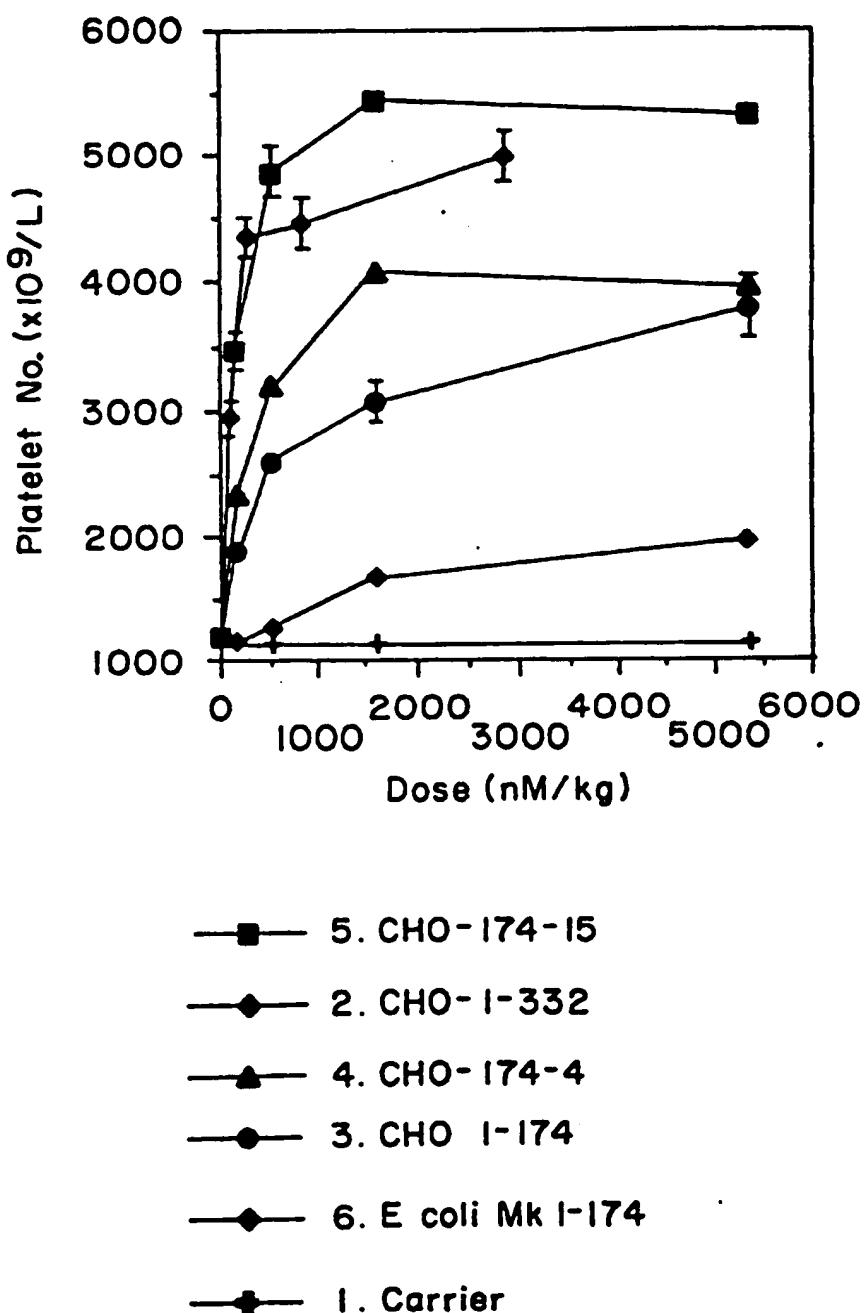
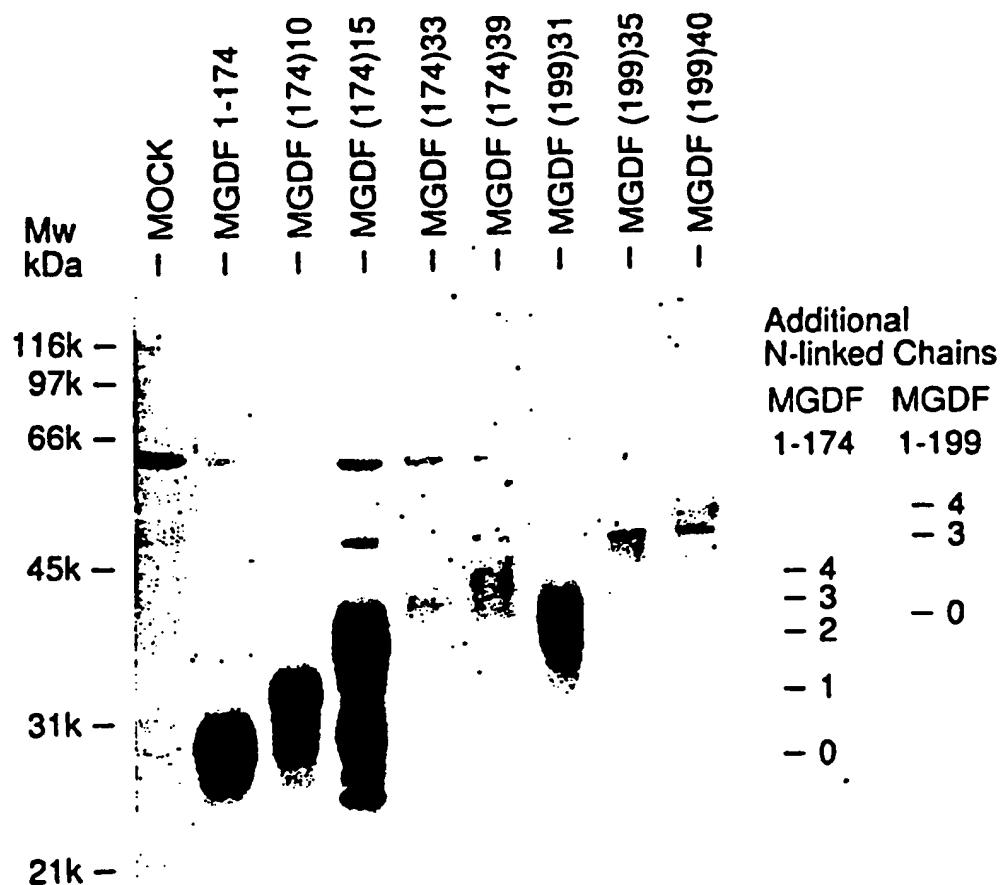


FIG. 10

FIG.11





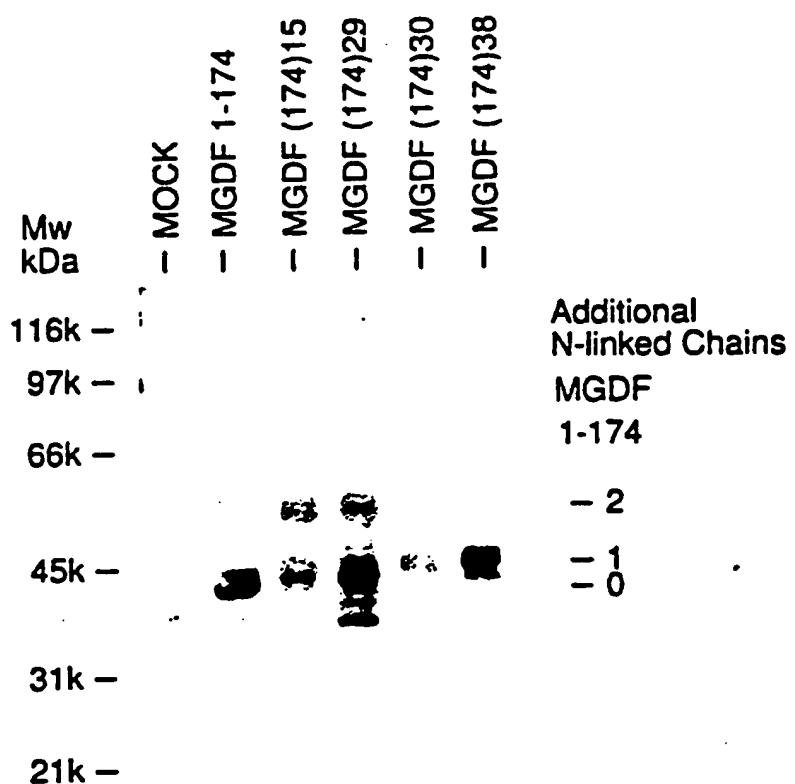


FIG.13